W Wardsby 3 50

589.93

LABORATORY MANUAL

IN

GENERAL MICROBIOLOGY

PREPARED BY THE

LABORATORY OF BACTERIOLOGY AND HYGIENE

MICHIGAN AGRICULTURAL COLLEGE

SECOND EDITION

NEW YORK

JOHN WILEY & SONS, INC.

LONDON: CHAPMAN & HALL, LIMITED

1921

PRESS OF
BRAUNWORTH & CO.
BOOK MANUFACTURERS
BROOKLYN, N. Y.

PREFATORY NOTE TO SECOND EDITION

Since the first appearance of this Manual in 1915, there have occurred some developments of fundamental importance in our conceptions of microbiological technic and laboratory procedure, as a consequence of which a revision of the text has become almost obligatory. Nearly all of our associates in the laboratory were connected with some branch of the Medical Corps during the War, and in their various capacities gained new viewpoints of certain phases of microbiological laboratory operations. Constant use of the Manual in our own laboratory and suggestions from a great number of sources, kindly furnished by teachers and even laboratory workers in various parts of the country, have induced us to attempt a revision with numerous corrections and many additions. The use of Fuller's Scale has been abandoned; the use of the autoclav rather than flowing steam in sterilization is recommended; the more recent views regarding reaction of media involving the determination of pH have been given consideration; the sections dealing with soil and water and sewage bacteriology have been almost completely rewritten; important alterations and additions have been made to the section on pathogenic bacteriology; vital additions have been made to the Appendix. It has been thought advisable to include in the pages preceding the first exercise a list of apparatus required by each student, also an outline or schedule for the use of the Manual in the beginning classes. Extensive changes in references have been made. New literature and new editions of texts demand that the instructor keep in touch himself and put his students in touch with the changes.

To Associate Professor F. W. Fabian is due full credit for efforts in making this revision possible, especially for the material relating to titration of media and pH determination. He has been ably assisted by Assistant Professor W. L. Mallmann, who has paid special attention to the exercises on water and sewage bacteriology. These men have given the guide a thorough trial in our student laboratory. Mrs. Zae Northrup Wyant has assisted in particular with the exercises on soil bacteriology, and Mr. I. F. Huddleson has made some corrections and additions to the section in pathogenic bacteriology and immunity. It is felt that the added exercises in this section will greatly enhance the value of the book. We are grateful to the research associates and assistants on our own staff, Mr. L. H. Cooledge, Dr. H. J. Stafseth, Mr. G. L. A. Ruehle and Dr. Robert Snyder, and to Prof. Arao Itano of the Massachusetts Agricultural College, as well as to the numerous other friends who have given us the advantage of their valuable experience with the Manual in their respective laboratories by means of helpful criticisms and suggestions. The inadequacies in the field of bacteriological laboratory guides in general and the imperfections and shortcomings of our efforts urge us to appeal for a continuation of the helpful attitude on the part of those using this guide.

Ward Giltner.

East Lansing, Michigan.
May, 1920.

PREFATORY NOTE TO FIRST EDITION

Laboratory instruction in bacteriology at the Michigan Agricultural College developed under the direction of Dr. C. E. Marshall. This laboratory guide represents the accumulated efforts of instructors working for a period in excess of a decade. To Assistant Professor L. Zae Northrup is due the credit for collecting and arranging the material presented as well as for preparing *de novo* many of the experiments and much of the supplementary matter. She has been assisted by Mr. W. L. Kulp. Dr. E. T. Hallman and Dr. L. R. Himmelberger have taken the responsibility for arranging the exercises relating to immunity, serum therapy and pathologic bacteriology. Great praise is due Dr. F. H. H. Van Suchtelen for introducing many new features into the laboratory work during the academic year 1912–13, and also Dr. Otto Rahn for his several years of admirable effort immediately preceding. Others whose influence has been felt in creating this guide and to whom credit is due are Professors W. G. Sackett, S. F. Edwards, L. D. Bushnell, C. W. Brown and W. H. Wright.

While some claim to originality may be made for this laboratory guide, it is to be expected that much of the material herein has been presented in various other manuals and perhaps in better form in many instances. The greatest effort has been made to make this a laboratory guide to General Microbiology, leaving the particular fields of dairy, soil, water, medical and other phases of bacteriology to special guides already in print or at present projected. The presentation of this manual to the public is in no way an

intimation that the special fields have not been admirably dealt with by others.

The subject matter given under Part I of this manual is primarily for the purpose of giving a working knowledge of laboratory methods used in the study of microorganisms. Molds, yeasts and bacteria are taken up in the order of their comparative sizes and studied as to their identification by morphological and cultural methods. It is presupposed that the student has a knowledge of these microorganisms acquired from preceding lectures in microbiology.

Part II consists of exercises demonstrating the various physiological activities of microorganisms.

Part III deals with applied microbiology. After the student has familiarized himself with the ordinary tools and technic, etc., as dealt with in Parts I and II, it is not necessary that he be burdened with minute, detailed instructions. We have had this in mind in preparing Part III.

No attempt has been made to compile an exhaustive list of exercises; the aim has been only to cover a wide range of activities under each different subject. In many cases, exercises have been taken directly, with few or no modifications, from laboratory manuals already in print. Credit has not been given directly; the list of references, however, includes all books from which material has been taken.

The purpose of this laboratory manual is to make the student more independent. Practically all directions for work to be done are contained in it; for this reason the work as assigned from day to day should be read over carefully *before beginning an exercise* and then followed step by step. Any desirable changes in directions may be indicated by the instructor.

WARD GILTNER,
Head of Department.

EAST LANSING, MICH.
Sept. 1, 1915.

CONTENTS

PART I

GENERAL MORPHOLOGICAL AND CULTURAL METHODS

PART II

PHYSIOLOGY OF MIGROORGANISMS

PART III

APPLIED MICROBIOLOGY

AIR MICROBIOLOGY

WATER AND SEWAGE MICROBIOLOGY

SOIL MICROBIOLOGY

DAIRY MICROBIOLOGY

PLANT MICROBIOLOGY

ANIMAL DISEASES AND IMMUNITY

APPENDIX

LABORATORY RULES

1. Do not bring coats, sweaters, hats, etc., into the laboratory and lay them on desks, etc.; hang them in the place provided for the purpose.

2. Before beginning and after finishing work, the top of the desks must be washed off with a liberal supply of 1–1000 mercuric chloride. This will destroy all micro-organisms and their spores and aid greatly in rendering aseptic technic possible. A large bottle of this disinfectant will be found near each desk.

3. Do not put string, paper, pencils, pins, etc., in the mouth nor moisten labels with the tongue while in the laboratory. Follow this practice outside of the laboratory also. Food should not be eaten in the laboratory.

4. Observe all possible cleanliness and neatness in the care of apparatus, desk, microscope, etc.

5. Apparatus must be kept inside the desks, but not cultures. Cultures must be kept at a constant temperature in the place fitted for this purpose.

The microscope and accessories must be returned to the case at the close of the work.

6. Water, gas, steam and electricity are to be turned off when not in use. This applies to the individual desks, large sinks, steam (including autoclav), hot-air sterilizers, etc.

7. Put all solid waste material, cotton, paper, matches, coagulated milk, etc., and waste liquid *which will solidify when cold* (agar, gelatin) into receptacles provided for that purpose, *not into the sinks.*

8. Apparatus, media, etc., should be removed from steam heaters, immediately after steaming.

9. No cultures are to be taken out of the laboratory without the permission of the head of the department.

10. All accidents, such as spilling infected material (pathogenic or non-pathogenic), cutting or pricking the fingers, must be reported at once to the instructor in charge.

Additional rules will be given if necessary, in conjunction with special exercises or technic.

11. 1–1000 mercuric chloride will not injure the skin if not used too often. Wash your hands in it thoroughly each time before you leave the laboratory to avoid carrying away undesirable organisms. **Use every precaution against infection.**

12. At the beginning of each laboratory period read over carefully the directions for the next exercise in order to understand its purpose and to make any necessary preliminary preparations.

13. Take careful notes on all observations made in the study of cultures and preparations made from them.

LIST OF APPARATUS NECESSARY FOR ONE STUDENT IN A LABORATORY

Number	Article
1	Apparatus, catalase
2	Baskets, wire, large
4	Baskets, wire, small
2	Bottles, glass, ordinary
2	Bottles, glass, stoppered
2	Brushes, test tube
1	Burette, 25 c.c. with tip
2	Clamps, burette
1	Cup, measuring, 1 l.
4	Dishes, Esmarch
1	Dish, evaporating
15	Dishes, Petri, ordinary
2	Dishes, Petri, medium
2	Dishes, Petri, large
14	Fermentation tubes
1	Flask, distilling, 500 c.c.
2	Flasks, Erlenmeyer, 1 l.
2	Flasks, Erlenmeyer, 500 c.c.
2	Flasks, Erlenmeyer, 375 c.c.
7	Flasks, Erlenmeyer, 200 c.c.
2	Flasks, Erlenmeyer, 100 c.c.
2	Flasks, Erlenmeyer, 75 c.c.
2	Flasks, Erlenmeyer, 50 c.c.
1	Flask, florence, 1 l.
1	Flask, florence, 500 c.c.
1	Flask, florence, 250 c.c.
2	Flasks, Roux
2	Forceps, coverglass
1	Forceps, ordinary
1	Funnel, large
1	Funnel, small, filling,
1	Graduate, 10 c.c.
1	Graduate, 100 c.c.
1	Jar, bacteria, 3.5 l.
1	Knife, potato
1	Lens, magnifying, 4×
1	Microscope and accessories
1	Needle, dissecting
1	Objective, dry, $\frac{1}{3}$ in. and $\frac{1}{7}$ in.
1	Objective, oil immersion $\frac{1}{12}$ in.
1	Ocular, Nos. 1, 3 and 4 or 5×, 10×.
2	Pinch-cocks
10	Pipettes, 1 c.c. grad.
4	Pipettes, 5 c.c. grad.
4	Pipettes, 10 c.c. grad.
2	Pipettes, 100 c.c. bulb
3	Racks, Petri-dish
2	Racks, test tube, wire
2	Rings, iron
4	Rings, moist chamber
2	Rods, glass
1	Ruler, celluloid, cm. inch
1	Scissors
6	Slides, concave
24	Slides, glass
2	Test glasses
250	Test tubes, ordinary
50	Test tubes, small

Number	Article	Number	Article
12	Test tubes, on foot, large	50	Cover glasses
1	Thermometer, 0°-110° C.	150 gms.	Gelatin
3	Tumblers	48	Labels
2	Wires, platinum	1 box	Matches
15 gms.	Agar agar	1 lb.	Meat
30 gms.	Albumen, egg	10 gms.	Meat extract
200 c.c.	Alcohol	3 pcs.	Paper, filter
1	Box, slide	6 pcs.	Paper, lens
250 c.c.	Cider, wort	1	Pen, drawing
2.5 yds.	Cloth, physician's	1	Pencil, wax
2 lbs.	Cotton	30 gms.	Peptone
		1 bar	Soap

Note.—Materials for stains and for test solutions and general apparatus forming a part of the laboratory equipment are not included in this list.

OUTLINE FOR USE OF MANUAL *

First Week

Monday	Check in desks. Put test-tubes to soak in cleaning solution.
Tuesday	Commence cleaning of glassware, following directions in the manual.
Wednesday	Continue cleaning of glassware.
Thursday	Prepare glassware for sterilization.
Friday	Continue preparation of glassware for sterilization. Start cleaning of cover-slips and slides.

Second Week

Monday	Complete cleaning of cover-slips and slides. All glassware should now be ready for use.
Tuesday	Demonstration of titration. Obtain 200 c.c. of cider, titrate and tube, placing about 8 c.c. in each tube. Sterilize.
Wednesday	Written quiz. Put 2.5 lbs. lean beef to soak in 1200 c.c. tap water. Place in refrigerator.
Thursday	Complete Ex. 6. Using 500 of unsterilized meat infusion, prepare nutrient broth, Ex. 7 as far as step 5.
Friday	Complete Ex. 7.

Third Week

Monday	Commence preparation of nutrient gelatin, Ex. 8. Complete through step 5. Start Ex. 46.
Tuesday	Complete Ex. 8. Tube 15 tubes of Dunham's Solution. Continue Ex. 46.

* This outline is intended as a suggestion to show how this manual may be used in the class room.

Wednesday	Start Ex. 9, complete through step 7. Continue Ex. 46.
Thursday	Complete Ex. 9. Tube 15 tubes of Nitrate Peptone Solution. Continue Ex. 46.
Friday	Ex. 5, "Preparation of Potato Medium." Continue Ex. 46. Make loop dilution plates of mixed mold culture pp. 49–52 and Ex. 37, step 1.

Fourth Week

Monday	Calibrate microscope—pp. 61–67. Measure prepared slides of diatoms for practice. Continue Ex. 46.
Tuesday	Using well-isolated colonies of the molds showing spores plate out, make transfers—Ex. 37, p. 107, step 5, *a*, *b*, *c*, and *d*. Make same transfers for two pure cultures furnished. Continue Ex. 46.
Wednesday	Observe, draw, and describe all twenty-four hour cultures. Continue Ex. 46.
Thursday	Make moist chamber culture of the 4 molds—Ex. 37, p. 107, step 5 *d*. Continue Ex. 46.
Friday	Make microscopic drawing from moist chamber cultures of germinating spore and mycelium.

Fifth Week

Monday	Make microscopic drawings from moist chamber of fruiting bodies.
Tuesday	Complete all drawings of mold cultures.
Wednesday	Written quiz.
Thursday	Begin Ex. 40, p. 112, starting both A and B.
Friday	Lecture on yeast. Isolate *Mycoderma cucumerina* from pickle scum by loop dilution plates.

FORM FOR WRITING UP EXERCISES IN THE NOTEBOOK

I. Object. A concise statement of what the exercise is intended to prove or demonstrate is to be given.

II. Apparatus. This includes everything with the exception of the every-day tools such as burner, platinum needles, etc.

III. Cultures. A brief morphological and cultural description characteristic of each organism should be given, also its occurrence and importance. Certain organisms are used for a certain purpose. If this purpose is not evident, ascertain from the references given why these particular organisms were used.

IV. Method. State briefly but clearly your method of procedure.

V. Results. Give your results in full. Tabulate data so that they may be comprehended at a glance. Results often may be tabulated as + and −. Plot curves whenever possible.

VI. Conclusions. Draw the conclusion which *your own* results warrant.

VII. Error. You may know that your results and the consequent conclusions are in error. If so, state what you consider to be the correct results and conclusions, noting any irregularities or abnormalities which may have occurred to change the results.

VIII. Practical Application. Apply the principles involved in the exercise to some practical purpose.

IX. References. Give the substance of the references placed at the end of each exercise in your own words and apply to the exercise in question. Do not copy verbatim.

Note. In writing up the notebook, details under II and IV should be omitted, only the headings are necessary.

PART I

GENERAL MORPHOLOGICAL AND CULTURAL METHODS

EXERCISE 1. CLEANING GLASSWARE

Glassware for use in microbiological laboratory work should be not merely clean, but *chemically* clean. Test tubes, Petri dishes, flasks, etc., are the receptacles used in the microbiological laboratory for containing the different nutrient substances upon which microorganisms are to subsist. Very frequently free alkali may be present on new glassware in sufficient quantity to prevent microbial growths in the nutrients contained therein. Prescott and Winslow in testing out different glassware say that, "The more soluble glassware yielded sufficient alkali to the medium to inhibit four-fifths of the bacteria present in certain cases."

Glassware which *looks* clean may have been used previously and should be given a thorough cleaning to rid it of possible traces of mercuric chloride, or other chemical having germicidal properties.

Follow directions carefully and clean all new and apparently clean glassware in the order given.

Cleaning New or Apparently Clean Glassware. All new glassware should first be treated with chromic acid cleaning solution* (*see appendix for all formulæ*) before proceeding with the directions for cleaning glassware.

Return used cleaning solution to the glass receptacle provided for the purpose. Do not throw it away. This

* Chromic acid cleaning solution will be designated hereafter merely as cleaning solution.

solution may be used until oxidized, i.e., until dark green in color.

Heat will facilitate the action of the cleaning solution.

Small amounts of organic matter adhering to glassware are oxidized by this solution, but will not disappear until removed by a suitable brush and cleaning powder.*

New Petri dishes and test tubes may conveniently be

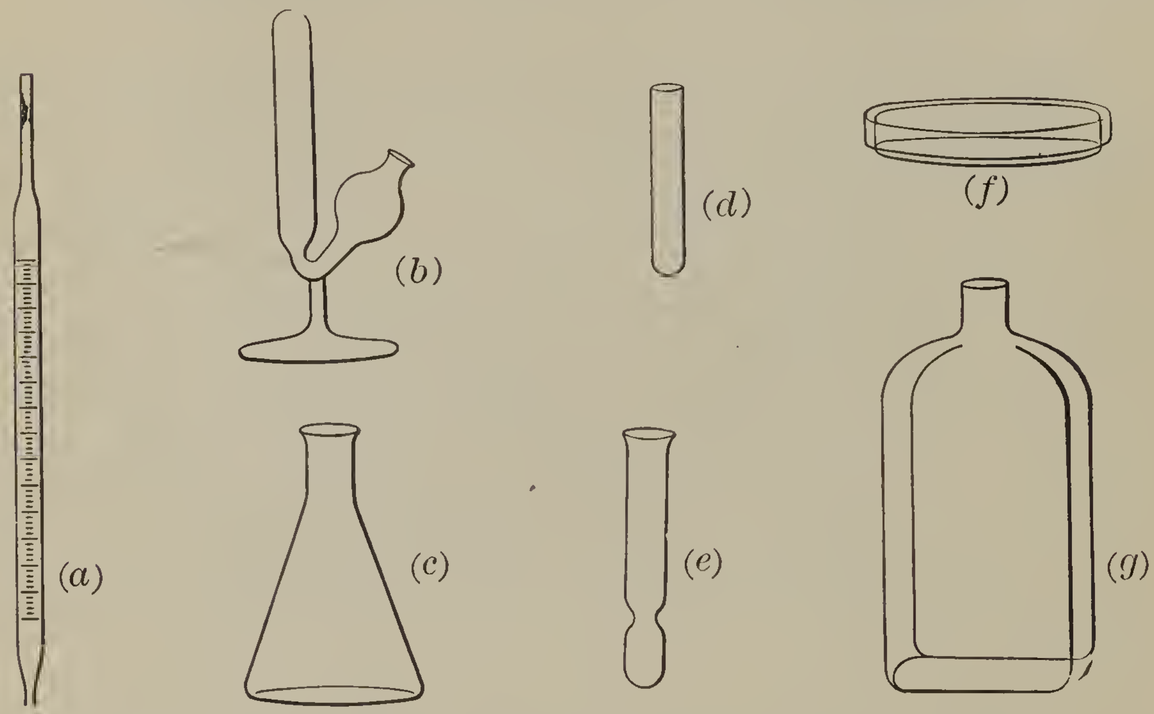


FIG. 1.—(*a*) Pipette, (*b*) Smith's Fermentation Tube, (*c*) Erlenmeyer Flask, (*d*) Test Tube, (*e*) Roux Tube, *f*) Petri Dish, (*g*) Roux Flask.

placed in a large glass jar, covered with cleaning solution and allowed to stand over night. Heavy glass jars will not stand heating in steam. New flasks may be partially filled with cleaning solution and placed in steam for fifteen minutes.

Test Tubes. New test tubes should be filled with cleaning solution, placed in a wire basket and heated for

* Any inexpensive fine-grained cleaning powder as powdered pumice stone, Bon Ami, etc., may be used.

at least fifteen minutes in the steam. After removing the cleaning solution from the test tubes:

1. Wash them in water with a test-tube brush, using cleaning powder if necessary.

2. Rinse with tap water till clean and free from cleaning powder.

3. Rinse with distilled water.

4. Drain.

5. Test tubes and other glassware, flasks, pipettes, etc., may be rinsed with alcohol to facilitate drying, then drained.

Flasks. After treating flasks with cleaning solution:

1. Wash them as clean as possible with tap water and a flask brush; use cleaning powder if necessary. (When using cleaning powder, empty all water out of the flask, wet the flask brush with tap water, dip it in the cleaning powder and then rub the soiled portions vigorously.)

2. Rinse with tap water till clear and free from cleaning powder.

3. Rinse with distilled water and drain.

Petri Dishes. After removing Petri dishes from the cleaning solution:

1. Wash them in water, using cleaning powder if necessary.

2. Rinse with tap water. (It is not necessary to use alcohol or distilled water.)

3. Wipe immediately with a clean physician's cloth.

Pipettes. 1. Place pipettes delivery end down, in a glass cylinder (graduate) in cleaning solution and allow them to stand over night. (Steam may break the glass cylinder.)

2. Pipettes which have been used should be washed immediately. Grease which cannot be removed with water should be treated with 10% NaOH* and then with cleaning solution.

3. Rinse with tap water, followed by distilled water.

* If strong NaOH stands in contact with a poor grade of glassware for a length of time, it etches the glass, leaving a blue haze which cannot be removed.

4. Rinse with alcohol. (Alcohol may be used repeatedly.)
5. Drain.

Fermentation Tubes. 1. Rinse with tap water.

2. Fill with cleaning solution and heat fifteen minutes in steam or allow to stand over night if more convenient.

3. Wash thoroughly in tap water, using a test-tube brush if necessary.

4. Rinse in distilled water and drain.

Cover-glasses and Slides. 1. Immerse the cover-glasses or slides, *one by one* in a 10% solution of sodium hydrate (NaOH) for thirty minutes only. This strength of NaOH will etch the glassware if left longer.

2. Wash *separately* in tap water, *handling with ordinary forceps.**

3. Put, *one at a time,* in cleaning solution, and leave over night as convenient.

4. Wash *separately* in water.
5. Immerse in *clean* alcohol (95%).
6. Wipe with a *clean* physician's cloth.
7. Store in clean Esmarch and deep culture dishes respectively, to keep free from dust.

Other Glassware. Some modification of these methods will be adaptable to nearly all glassware.

Note 1. Glassware containing liquefiable solid media is best cleaned by heating and pouring out the material while in liquid condition, then treating as above. (Solid media when liquefied by heat should never be thrown in the sink, as it will solidify when cold and clog up the traps and drains.)

Note 2. Flasks, test tubes, Petri dishes, etc., *containing cultures,* must be heated one hour in flowing steam *before cleaning.*

Cultures containing spores should be *autoclaved previous to cleaning.*

Note 3. If cultures or media have become dry, add water before heating.

Especial care must be used in cleaning glassware in which mercuric chloride or any other disinfectant has been used.

*** Always handle cover-glasses and slides with forceps.**

STERILIZATION

Sterilization consists in the destruction of all forms of life. It may be effected by various agents. As applied to the practical requirements of the bacteriological laboratory many of those agents such as electricity, sunlight, etc., are of little value and are limited in their applications; others are so well suited to particular purposes that their use is almost entirely restricted to such applications.

The Two General Methods of Sterilization are:

A. Physical.

1. Plasmolysis or Plasmoptysis.
2. Desiccation.
3. Heat—(*a*) dry heat; (*b*) moist heat.
4. Light.
5. Filtration.
6. Dialysis.
7. Comminution.

B. Chemical.

1. Disinfectants, etc.

A. PHYSICAL

I. Concentrated solutions destroy microorganisms by withdrawing water from their cells (*plasmolysis*), e.g., in the preservation of food by concentrated salt or sugar solutions.

Microorganisms accustomed to a concentrated nutrient substrate may suffer *plasmoptysis* (bursting of the cell) if placed in a less concentrated medium.

In either case, if they are subjected gradually to the changing conditions, death is delayed or prevented.

II. Desiccation is destructive to many microbes, especially to those which do not form spores. For example, *B. radicicola* is very sensitive to desiccation on the ordinary cover-glass or on cotton.

III. Sterilization by Dry Heat.

1. Sterilization in a naked flame.
2. Sterilization in an ether flame.
3. Sterilization in a muffle furnace.
4. Sterilization by hot air.

1. *Sterilization in a Naked Flame.* (*a*) The simplest means of sterilizing a metal instrument is to heat it to redness in a flame. This method is always adopted for sterilizing platinum, copper, etc., wires and iron and nickel spatulas, forceps, etc.

A platinum needle should always be carefully dried before sterilization, by holding it near the flame. This avoids sputtering, which scatters microorganisms, especially if moist material, e.g., fat or protein, on the needle is immediately thrust into the flame.

(*b*) An instrument may be sterilized by flaming it, i.e., by passing it rapidly through a hot flame. This method is useful for instruments, etc., having polished surfaces devoid of creases in which microorganisms might escape destruction, e.g., knives, glass rods, handles of platinum needles, mouths of test tubes, flasks, pipettes, etc.

(*c*) Deep wounds are sterilized by cautery with an instrument heated to a dull red heat.

2. *Sterilization in an Ether Flame.* In an emergency, small instruments, needles, etc., may be sterilized by dipping them in ether or absolute alcohol and after removal lighting the adherent fluid and allowing it to burn off the surface of the instruments. Repeat the process. It may then be safely assumed that the apparatus so treated is sterile.

3. *Sterilization in a Muffle Furnace.* Porcelain filter candles are sterilized by heating them to white heat in the muffle furnace. This method of sterilization cannot be applied to porcelain filters with metal fittings, such as Berkefeld filters.

The destruction of autopsied animals and accumulated

wastes of the laboratory is also best accomplished in this manner.

4. *Sterilization by Hot Air.* Exposure to hot air is the usual method of sterilizing all glassware, instruments with metal handles, etc., but it is not suitable for organic substances, with the exception of wool, cotton and paper.

To insure efficient sterilization, the prepared glassware,

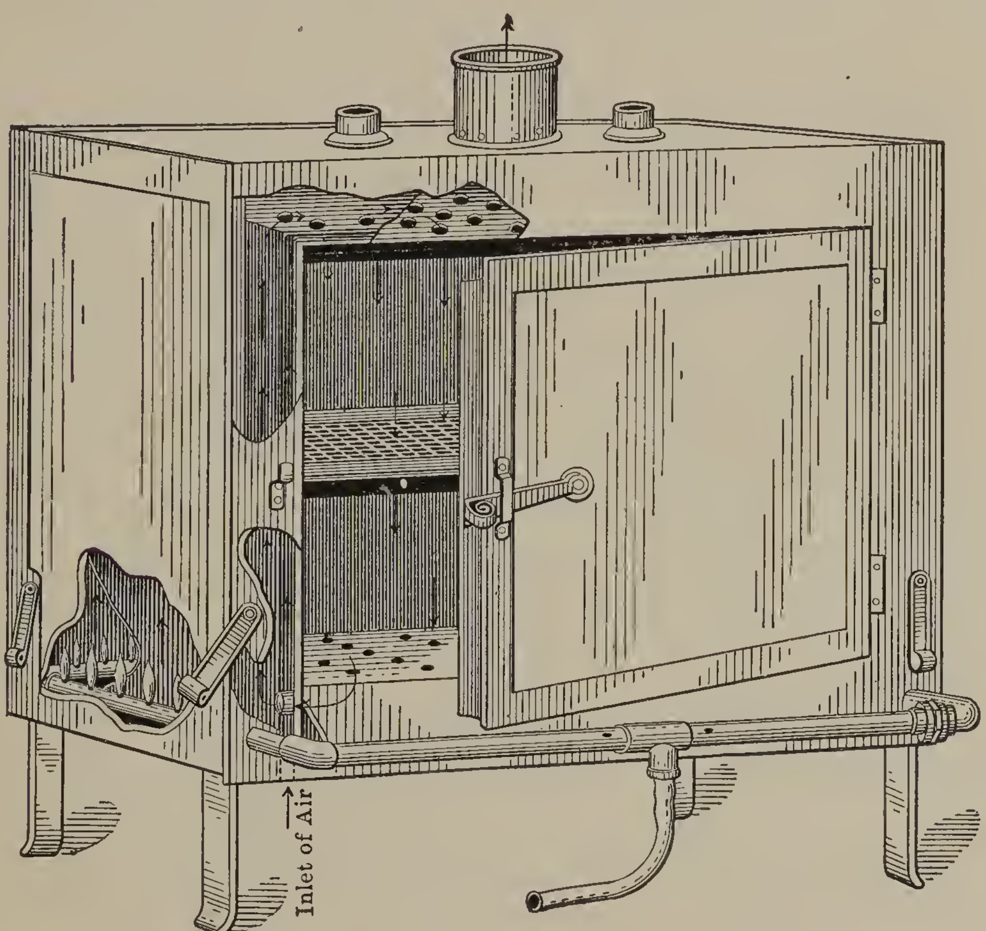


Fig. 2.—Hot Air Sterilizer.

etc., must be placed in a gas or electrically heated oven (containing a thermometer registering over 200° C.) whose temperature is maintained at approximately 150° C. for one hour, or 180° C. for ten minutes. The oven must be allowed to cool down to 60° C. before opening the door to avoid the breaking of glassware by cold-air currents. Cotton, wool, and paper are slightly scorched at this temperature.

Apparatus must be absolutely clean and *dry* before being sterilized.

IV. Sterilization by Moist Heat. Sterilization by moist heat may be effected in one of four ways:

1. By continuous or discontinuous heating at low temperatures (56°–80° C.).

2. By continuous or discontinuous heating in water at 100° C.

3. By continuous or discontinuous heating in flowing steam at 100° C.

4. By one heating in superheated steam (steam under pressure) at temperatures above 100° C., generally 115° C. (about 10 lbs. pressure) or 120° C. (about 15 lbs.).

1. *Sterilization by Continuous or Discontinuous Heating at Low Temperatures.* Some substances used as culture media, being rich in volatile or otherwise chemically unstable substances, cannot be heated to 100° C. without a marked alteration (e.g., coagulation) and to some extent a destruction of their properties; blood serum, for example.

Pasteur showed that such media can be better sterilized by heating them at a low temperature (55°–60° C.) for a long time than at a high temperature (70° C. or even 100° C.) for a short time. In this process, heat is not applied directly, as a rule. Control of the temperature is ordinarily accomplished by means of water heated to the degree desired.

Prolonged heating at a low temperature constitutes **pasteurization.** In practice, however, it is found that in order to kill all organisms pasteurization must be combined with the method of discontinuous heating devised by Tyndall. Albuminous media subjected to the Tyndall method must be incubated finally at 37° C. for forty-eight hours to eliminate all contaminated specimens.

2. *Sterilization by Continuous or Discontinuous Heating in Water at* 100° C. (*a*) *Continuous Heating.* Water at 100° C. destroys the vegetative forms of bacteria almost

instantaneously, and spores in from five to fifteen minutes ordinarily, although many spores of resistant species are not killed by several hours' heating at 100° C. Water suspected of sewage contamination may thus be rendered safe for drinking purposes simply by boiling for a few minutes.

This method is applicable to metal instruments, syringes, rubber stoppers, rubber and glass tubing, and other small apparatus.

(*b*) *Discontinuous Heating.* (Tyndall method.) Tyndall observed that certain resistant forms found in an infusion made from hay were not destroyed by heating the infusion at 100° C., once, even when the temperature was sustained for a prolonged period, yet by boiling it for a short time on three successive days all living organisms were destroyed. His theory was that by heating at 100° C., the vegetative forms but not the spores were killed. The latter germinate as the fluid cools and are killed during the second heating. A few spores, however, escape destruction at the second heating; these will have germinated by the time the third heating is due. After the third heating sterilization is accomplished.

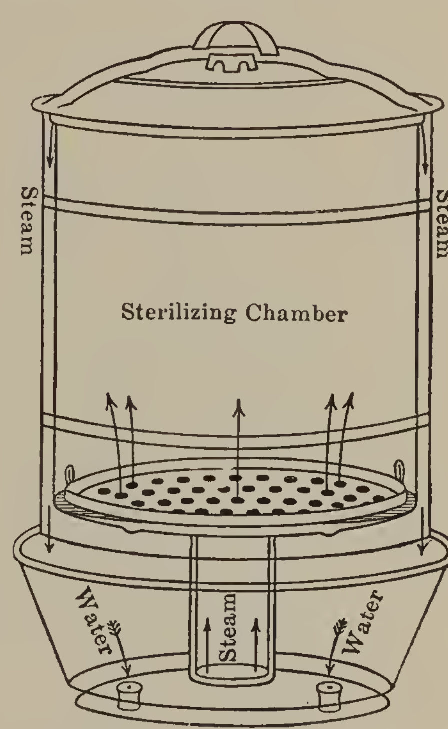


Fig. 3.—Arnold Steam Sterilizer.

The explanation now given, however, is that the resistance of spores is gradually lowered under the influence of repeated heatings. This principle of heating, on three successive days, a medium to be sterilized is now known as the **Tyndall method of sterilization.** In general laboratory practice, steam is used instead of water at 100° C., but this necessitates special apparatus, whereas water lends itself readily to the means at hand.

The physical nature or bulk of the medium, the extraordinary resistance of the spores of certain bacteria or both in combination, may require that this intermittent heating be carried on over a longer period of time, i.e., four, five, six, etc., days in succession for the same or a longer period each time, or that the period between intermittent heatings be lengthened from twenty-four hours to forty-eight hours.

Tyndall's method is valuable in that media of delicate composition may be sterilized without producing undesirable changes, such as are often produced by the high temperature of the autoclav.

3. *Sterilization in Flowing Steam at* 100° C. *Continuous or Discontinuous.* (*a*) *Continuous Heating.* Simple boiling or exposure to steam at 100° C., even though the exposure be prolonged, is not a reliable method of sterilization. When microorganisms have been dried, their resistance to the effects of heat is much enhanced, and especially is this the case when they are mixed with substances of a colloidal nature. Certain resistant forms of protoplasm known as spores may not be destroyed by one heating to 100° C., even when the temperature has been maintained for several minutes.

(*b*) *Discontinuous Heating.* General use for the sterilization of media.

This principle of sterilization advanced by Tyndall finds its widest application in bacteriological work with the use of flowing steam. High-pressure steam may be utilized to good advantage if a central heating station is available. The Arnold sterilizer makes use of steam for the sterilization process and lends itself readily to both the continuous and discontinuous method (Fig. 3).

4. *Sterilization by Superheated Steam* (under pressure and therefore above 100° C.). Water, syringes, surgical dressings, bedding, india-rubber apparatus, filters, old cultivations, culture media, etc., not injured by high tem-

peratures, may be more quickly sterilized by heating in steam under pressure.

Exposure to steam at a temperature of 115° C. for twenty minutes is in most cases sufficient to insure sterilization,

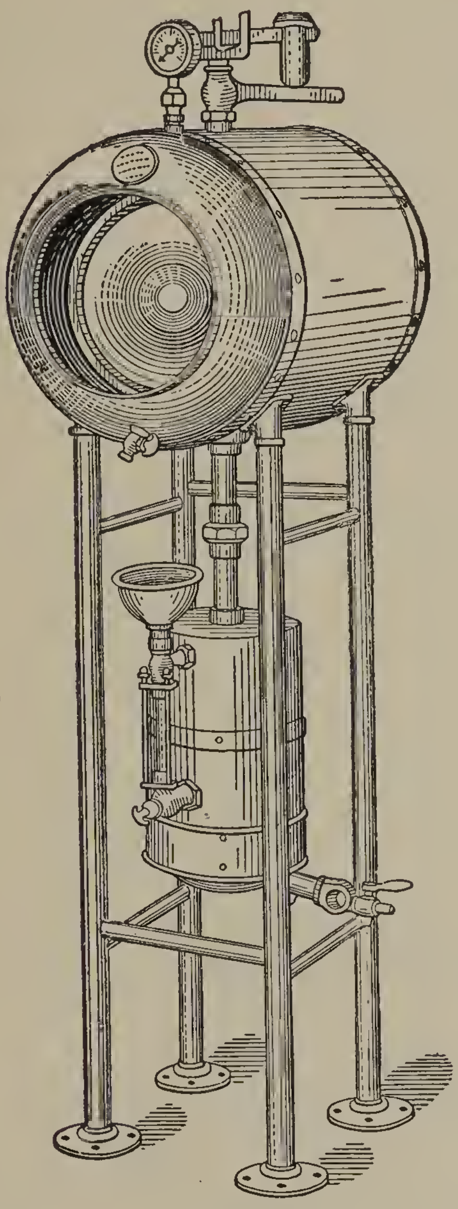


FIG. 4.—Autoclav, Horizontal, for Steam or Gas.

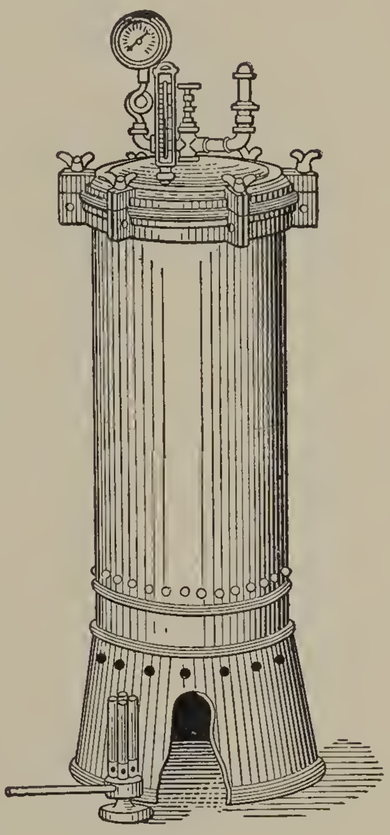


FIG. 5.—Autoclav, Vertical, for Gas Only.

but some media, potato for instance, require a temperature of 120° C. for ten to fifteen minutes. It is now realized that media subjected to this high temperature undergo hydrolytic changes which render them unsuitable for the cultivation of more delicate microorganisms. Sterilization

in the superheated steam is carried on in a special apparatus called an **autoclav,** which may be so constructed as to run by direct or indirect steam. The latter is the more desirable for the sterilization of media.

V. Sterilization by Light. Light seems to act by producing powerful chemical germicides, probably organic peroxides, in the medium surrounding the bacteria. Certain rays of light, the blue, violet and ultraviolet in particular are destructive to living cells. It is to these rays that sunlight owes its disinfecting action. Practical use has been made of the ultraviolet rays in water sterilization by employing the Cooper–Hewitt mercury vapor lamp having a quartz instead of a glass tube, as these rays do not pass through glass.

VI. Sterilization by Filtration. Sterilization may be effected by the filtration of gases or liquids through materials which will retain microorganisms.

The best example of the filtration of gases is the use of cotton plugs in flasks and tubes containing microorganisms. The cotton is porous enough to allow the necessary interchange of gases but will allow neither dust nor foreign microorganisms to enter. The sterilization of air or other gases if forced through cotton would depend upon the thickness of the cotton layer and also upon the force which was exerted.

Certain fluids used in bacteriological work cannot be subjected even to a moderate amount of heat without profoundly altering their nature. In order to make such a fluid sterile, it is passed through a cylindrical vessel, closed at one end like a test tube, and made either of porous "biscuit" porcelain, hard burnt and unglazed (Chamberland filter) or of kieselguhr, a fine diatomaceous earth (Berkefeld filter) and termed a **bougie** or **candle**. (Fig. 70, p. 318.)

The pores of the finer filters are so small that, while liquids and solids in solution pass through, microorganisms are retained and the liquid passes through in a germ-free

condition. Pasteur in his early work utilized plaster plates as the filtering medium, but as a result of Chamberland's researches, porous porcelain now supersedes plaster. Finely shredded asbestos packed tightly in a Gooch crucible will serve as a bacterial filter provided the layer of asbestos is sufficiently thick. The rate of filtration is usually very slow because the pores of the filter are so very minute; therefore to overcome this disadvantage either aspiration or pressure is generally employed to hasten the process. This method may not exclude filterable organisms.

VII. Sterilization by Dialysis. In one of the more recent methods devised for the preparation of antirabic vaccines the vaccine is prepared by placing the virus (spinal cord of a rabid rabbit) in a collodion sac and dialyzing it in running distilled water. The living virus is destroyed, yet its immunizing properties are retained unimpaired. Quite the opposite effect may be obtained under somewhat different circumstances. If a collodion sac containing a suspension of a pathogenic organism be placed in the body cavity of a susceptible animal the organisms within the sac thrive, being nourished by the body fluids which diffuse through the semi-permeable membrane.

Cumming, J. G.: Rabies—Hydrophobia. A study of fixed virus, determination of the M. L. D., vaccine treatment (Högyes, Pasteur, and dialyzed vaccine), and immunity tests. Journal of Infectious Diseases, Vol. XIV (1914), pp. 33–52.

VIII. Comminution or the actual crushing of the microbial cells is resorted to for demonstrating intracellular enzymes. Shaking or a trembling motion may be destructive to microbial cells, bacteria breaking up into fine particles.

B. CHEMICAL

I. Sterilization by Disinfectants. Sterilization by disinfectants has but limited use in bacteriological work. The amount of disinfectant necessary to destroy existing organisms in a nutrient medium is greater than the amount necessary to inhibit multiplication of an organism which may subsequently be used as an inoculum; the medium is therefore rendered useless.

1. Disinfectants may be used for any apparatus which will not come in direct contact with culture media or with the organisms under investigation. Fixed non-volatile disinfectants must be employed, since the vapors given off by volatile compounds hinder the growth of organisms on culture media.

2. Disinfectants are in general use for sterilizing the hands, woodwork, for washing out vessels and sterilizing instruments during inoculation and other experiments.

As an example, 1–1000 mercuric chloride, 1.5% formalin, 5% phenol, 2% compound solution of cresol, etc., are cheap and adaptable in many cases. Tincture of iodin is valuable for painting wounds.

The common *soaps*, and more particularly green soap, have a slight germicidal value, and this in conjunction with their solvent action upon fats and protein, and the mechanical cleansing which accompanies their use, justifies assigning them an important place among the chemical disinfectants.

Disinfectants used for sterilizing the skin before collecting pus, blood, etc., from the living subject must be carefully removed by washing the part well with alcohol before collecting material, otherwise the presence of the disinfectant would materially interfere with the subsequent growth of organisms in the culture.

3. Disinfectants are also added to sterile filtrates which are no longer required as culture media. For this pur-

pose a small quantity of some disinfectant (such as thymol or camphor) which is without chemical action on the constituents of the fluid is selected.

An amount of carbolic acid (0.5%) or other chemical is frequently added to vaccines, bacterins, serums, etc., for preservative purposes.

4. Disinfectants are sometimes used to sterilize a culture when the products of the microorgansims are under investigation. Chloroform, ether, toluol, oil of garlic or mustard, etc., which may be driven off afterward by evaporation, are among the most useful in this connection.

II. Sterilization by Antiseptics. Chemical reagents such as belong to the class known as antiseptics, i.e., substances which inhibit the growth of, but do not destroy bacterial life, are obviously useless.

REFERENCES

Eyre: Bacteriological Technic. Second Edition (1913), pp. 26–48.
Besson: Practical Bacteriology, Microbiology, and Serum Therapy (1913), pp. 3–27.
Marshall: Microbiology. Second Edition, pp. 116–119.
Euler: General Chemistry of the Enzymes (1912), pp. 118–123.

EXERCISE 2. PREPARATION OF GLASSWARE FOR STERILIZATION

The mouths of test tubes, fermentation tubes, pipettes, etc., are ordinarily plugged with cotton before sterilization. For this purpose cotton is ideal as it is cheap and adaptable, serves to filter out microorganisms from the air, while allowing the ready diffusion of gases, and after once used it may be burned.

Paper (ordinary newspaper) may be used to wrap glassware as Petri dishes, deep-culture dishes, pipettes, etc., which one wishes to store in a sterile condition and for which cotton is not adaptable.

Glassware is sterilized for the purpose of destroying

microorganisms present on its surface and in or on the cotton or paper used respectively for plugging or wrapping. After sterilization the cotton and paper serve to prevent microorganisms from entering and contaminating the sterile utensils.

Dry heat, though not as effective a germ destroyer as moist heat, is more adaptable to the sterilization of empty culture flasks, pipettes and other glassware. Hot-air sterilization not only accomplishes the sterilization of the glassware, cotton plugs, etc., but " sets " the plugs so that they may be handled with greater facility.

All glassware must be absolutely clean and dry or contain *traces of alcohol only* before preparing for sterilization; otherwise sterilization cannot be accomplished. If considerable moisture is present in test tubes, flasks, etc., it will not evaporate during the hot-air sterilization process, and it is very evident that the temperature of such moist portions of the glassware will not reach or at least will not exceed 100° C.

Directions. Test tubes and **flasks** are plugged with cotton. The ordinary forceps are used for this purpose. (A glass rod may also be used.) A small piece of cotton is grasped on the edge with the forceps and inserted in the mouth of the test tube. Plugs should project into test tubes from 3 to 4 cms., and from 3 to 5 cms. into the neck of flasks, according to the size of the flask. Only an amount of cotton should project out of the mouth that is sufficient to protect the outward turned portion (lip) of the test tubes or flasks from dust. A " Christmas-tree " effect is to be avoided. Plugs should not be so tight as to be removed with difficulty, nor so loose as to offer no resistance to removal. A little experience will suffice to demonstrate the amount of cotton to use and the firmness with which the plug should fit.

Cotton plugs for test tubes, flasks, etc., may be *rolled.* This kind of plug is more stable and may be used several

times. Have the instructor demonstrate the method of rolling.

For hot-air sterilization, test tubes plugged with cotton may be tied in large bundles or placed in wire baskets

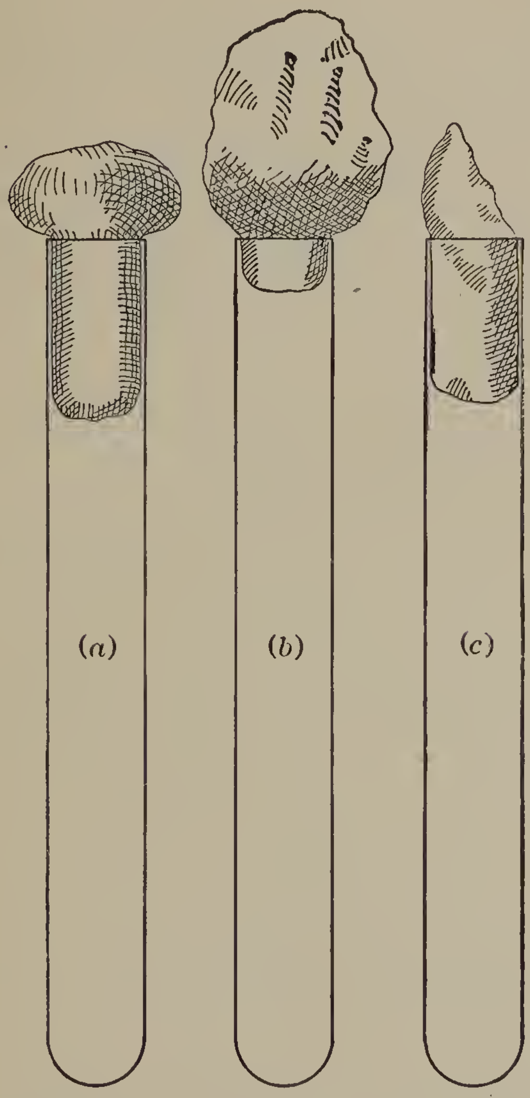


FIG. 6.—(*a*) Proper Plug; (*b*) Plug too Shallow and too Loose, too Much Projecting; (*c*) Plug too Loose, too Little Projecting.

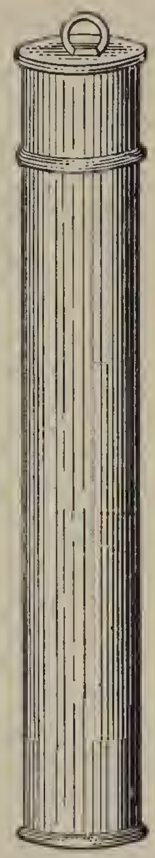


FIG. 7.—Copper Cylinder for Sterilizing Pipettes.

(never in agate cups), *cotton plugs up*. A few test tubes should not be placed in a large wire-basket or in a wire test-tube rack, as it is necessary to economize space in the hot-air sterilizer.

Petri dishes are wrapped separately in paper and tied together in sets of three. One sheet of newspaper makes

four papers of proper size for wrapping Petri dishes and is inexpensive.

Three or more Petri dishes may be wrapped together *if all are to be used at the same time.* Mark each plainly with the desk number.

Pipettes. Place a piece of cotton in the bottom of a test tube, plug the top only of the pipette with cotton (*not too tightly*), leaving but little of the cotton projecting out. Wrap a small portion of cotton around the lower third of the pipette, insert the pipette into the test tube until the tip rests on the cotton, making the cotton wrapping serve as a plug for the tube.

Wrap pipettes so prepared in paper (one layer) and tie and mark them plainly with the desk number.

A covered metal case is often used for holding pipettes to be sterilized. The upper end of the pipettes are plugged with cotton, the pipette inserted in the case, the open end of the case plugged with cotton, and the cover replaced. (This latter method is not recommended for the new student, as the necessity of careful technic in removing a sterile pipette from the case without contaminating those remaining is difficult to impress upon him).

Fermentation tubes are plugged with cotton as directed for test tubes; the cotton plug should not project into the bulb.

Deep culture dishes are wrapped singly in paper as directed for Petri dishes.

Slides and cover-glasses are generally sterilized by flaming, but *only as needed.*

NUTRIENT MEDIA

" Chemically, like all other living cells, microorganisms consist of organic and inorganic nitrogen and mineral salts; it is therefore necessary in order to grow a microorganism, that these three classes of substances be made available, together with oxygen, which is an essential to the life of all

living structures. Finally a certain amount of moisture is absolutely necessary." (Besson.)

A food prepared for the growth of microorganisms is given the general term **nutrient medium.** A large number of microorganisms will grow readily in or upon easily available nutrient media, as milk, bouillon, etc. Some microorganisms have widely differing food requirements and need for growth nutrient media differing widely in their composition.

However, there are a few general rules that must be applied in the preparation of all nutrient media for the use of microorganisms. These are briefly, that: Every culture medium must—1. Contain substances necessary for growth. 2. Be of suitable reaction. 3. Be contained in vessels which afford protection from contamination from without.

Classification of Nutrient Media. Culture media may be classified as:

I. *Natural Media*—as occurring in nature, e.g., milk, potato and other vegetables, meat and meat products, blood and blood serum, egg, soil, etc.

II. *Prepared media,* i.e., made in the laboratory. These are:

(*a*) Of unknown chemical composition; e.g., nutrient agar, gelatin, etc.

(*b*) Synthetic; i.e., chemical composition known, e.g., Giltay solution for denitrifying organisms.

Or as:

I. *Liquid Media.* These include:

A. Media made from animal tissue and fluids, e.g., nutrient broth, serum broth, carbohydrate broths, milk, blood, nitrate peptone solution, Dunham's solution.

B. Media made from vegetable tissue. Among these are: Malt extract (germinated barley), beer wort, yeast extract, hay infusion, natural fruit juices, wines (fermented fruit juices).

C. Synthetic media.

II. *Solid Media.* These may be classified as:

A. Liquefiable, e.g., nutrient agar, nutrient gelatin.

B. Non-liquefiable, including: 1. Media liquid in a natural state but which, once solidified, cannot be liquefied by physical means, e.g., media prepared from albuminous fluids and tissues such as egg, blood serum, etc., or synthetic media solidified with sodium silicate.

2. Media which are solid in the natural state, e.g., vegetable media such as potato, carrot, banana, etc.

EXERCISE 3. TITRATION OF MEDIA

At present there are three methods used to determine the reaction of bacteriological media. One method is by titrating with a known strength of acid or alkali, which gives the value of the total quantity of acid or alkali present. The second method is to determine the intensity of the hydrogen-ion by means of "buffer solutions" and standard indicators. The latter method has been made practical for laboratory use by Clark and Lubs and is coming into common use in laboratories. The third, and most accurate, is the hydrogen electrode method. The first method is the one described in this exercise. The second method is described in detail in the Appendix.

Procedure. 1. Adjust 45 c.c. of distilled water to a faint but distinct pink with N/20 NaOH, using phenolphthalein as indicator.

2. Then add 5 c.c. of medium to the distilled water.

3. Titrate again with N/20 NaOH and record results.

4. Compute and record the reaction of the medium in percentage normal solution, which is the number of cubic centimeters of normal acid or alkali required to neutralize 100 cubic centimeters of the medium, using phenolphthalein as indicator expressed as percentage of normal. Thus, a normal solution is 100% normal.

5. Alkaline media are denoted by placing a minus sign and acid media by placing a plus sign before the percentage of normal solution required to neutralize.

Example. Measure 45 c.c. of distilled water into evaporating dish and add enough N/20 NaOH from burette to bring to desired color. Then take reading of burette and titrate the medium.

Burette reading after titrating medium $=5.4$ c.c.
Burette reading before titrating medium $=2.0$ c.c.
No. of c. c. N/20 NaOH required to neutralize the acid in 5 c.c. of medium $=3.4$ c.c.

The amount needed for the one liter can be easily computed by direct proportion as follows:

$$3.4 \text{ c.c.} : 5 = X : 1000$$
$$5X = 3400$$
$$X = 680 \text{ c.c. of N/20 NaOH.}$$

Since 680 c.c. of N/20 NaOH would dilute the medium too much, N/1 is always used. As a normal solution is twenty times the strength of a N/20 solution, it would take 1/20 of 680 or 34 c.c. of N/1 NaOH to neutralize 1000 c.c. of the medium.

To express this in terms of percentage normal solution required to neutralize, we have 34 c.c. N/1 NaOH per 1000 c.c. or 3.4 c.c. per 100 c.c. which is usually written $+3.4\%$ normal.

If the medium is alkaline, N/20 HCl is used instead of N/20 NaOH and the calculation is the same but the titre would be written -3.4% normal, which means that every 100 c.c. of medium would require 3.4 c.c. of N/1 HCl for neutralization.

Note. If N/10 normal solution instead of N/20 is used for titration, use 10 c.c. of medium and 40 c.c. of water.

Fuller's Scale is sometimes seen in literature and is defined as the number of cubic centimeters of normal acid or alkali present in 1000 cubic centimeters of the medium, using phenolphthalein as indicator. Thus, if it took 17 c.c. of N/1 NaOH to neutralize 1000 c.c. of medium, it would be written $+17°$ Fuller's scale.

Notes. A solution is said to be *normal* when it contains 1 *gram equivalent* of an acid or base in 1 liter.

A gram equivalent of an acid or a base is that quantity which is equivalent to or will neutralize 1 gram molecule of a *mono*-basic acid or of a *mon*-acid base.

The advantage of the system is that 1 c.c. of any normal

solution will exactly neutralize or be exactly equivalent to 1 milligram equivalent of any acid or base. (Noyes, Wm. A., Textbook of Chemistry, 1913, p. 184.)

CIDER, VINEGAR, WORT, AND FRUIT JUICES

For the cultivation of yeasts and molds partly fermented or unfermented (fresh) fruit and vegetable juices or extracts are frequently employed. These media are always more or less acid in reaction, for which reason they are not adapted to the cultivation of most bacteria. Most yeasts and molds, however, are acid tolerant or actually utilize organic acids as a food. Frequently these substances are highly colored, demanding the use of a greater dilution in titration, e.g., 1 c.c. to 49 c.c. of water. Aside from the change in calculation made necessary by the lessened amount of medium employed, the procedure for titration is the same as that described above. It is very essential that heating be avoided in the titration of these media, since they contain volatile acids or other organic substances.

MILK

Milk is valuable as a nutrient medium for microorganisms because: It is a natural nutriment and almost ideal for a large number of microorganisms. Its composition, averaging 3.40% fat, 3.50% casein and albumen, 4.50% milk sugar, 0.75% ash, 87.75% water, is an evidence that it furnishes food in an excellent form for most microorganisms.

The biochemical activities of many bacteria reveal themselves definitely in the changes which milk, especially litmus milk, undergoes. Many of these changes are seen macroscopically. Some of these are:

(*a*) **Acid Production.** The lactose, $C_{12}H_{22}O_{11}$ (milk sugar), is first inverted, forming two hexose molecules, 1 mol. dextrose and 1 mol. galactose.

$$C_{12}H_{22}O_{11}+H_2O=2C_6H_{12}O_6.$$

And each molecule of hexose yields two molecules of lactic acid:

$$\text{hexose} = \text{lactic acid.}$$

$$C_6H_{12}O_6 = 2CH_3CH(OH)COOH.$$

The blue litmus is turned red.

(b) **Alkali Production.** Litmus becomes darker blue. This change very often accompanies peptonization.

(c) **Reduction (Decolorization of Litmus).** This is due to the reduction of the coloring matter (litmus). Many microorganisms secrete enzymes which produce hydrogen. The hydrogen combines with the litmus, reducing it to its leuco-compound (colorless). (Methylen blue becomes colorless under like conditions.) That this is a reduction and not a destruction may be demonstrated by shaking the decolorized culture with a few cubic centimeters of hydrogen peroxid. The bacteria which decolorize the litmus also reduce the hydrogen peroxid to H_2O and nascent oxygen which reoxidizes the reduced litmus (showing by the reaction of the milk the type of microorganisms present). Reoxidation takes place slowly under natural conditions. Reduction may take place when milk is acid; alkaline or neutral.

(*d*) **Curdling through Acid Production.** The casein, like most proteins, is **amphoteric,** i.e., it is capable of reacting both as a weak acid and a weak base. The otherwise insoluble casein is found to be in the milk in a partially dissolved state (colloidal), due to its combination with the calcium salts: the calcium that was formerly combined with the casein, through the production of acid by certain microorganisms, now combines with the lactic acid; as a result the casein precipitates, causing curdling (coagulation). Litmus is turned decidedly red. Milk having an acid curd will titrate above +5% normal

(*e*) **Rennet Curd.** Coagulation may also take place when the medium is neutral or only slightly acid. This pro-

duction of curd is due to a rennet-like enzyme produced by microorganisms, and is similar to the action of the rennet used to curdle milk in cheese factories.

Many spore-forming species are found under the group of rennet-producing organisms. Rennet curd is usually followed by peptonization.

(*f*) **Peptonization.** The curd produced by acid or rennet-forming microorganisms may gradually disappear, leaving only a whey-like liquid. This is caused by certain bacteria which produce proteolytic enzymes that digest the curd and render it soluble. This liquefaction of solid proteins like gelatin, fibrin, boiled egg white, milk curd, etc., is due to two groups of enzymes, *pepsin* and *trypsin*.

The pepsin of the animal body acts only in an acid medium (present in the stomach).

The trypsin of the animal body acts only in alkaline medium (present in the intestine).

The pepsin- and trypsin-like enzymes produced by microorganisms cannot be thus separated by their activity in a medium of certain reaction; this varies with the species of microorganism and with environmental conditions. Peptonization of milk usually takes place in a neutral, slightly alkaline, or more infrequently slightly acid reaction.

Some organisms peptonize milk without forming a rennet curd.

(g) **Gas Production.** This is characterized by the formation of gas bubbles in the milk, and is generally accompanied by the formation of acid curd. Very commonly the curd shrinks, causing **extrusion of whey.**

EXERCISE 4. PREPARATION OF LITMUS MILK

Apparatus. Fresh separated or skimmed milk; titration apparatus; N/20 NaOH; phenolphthalein (indicator); 5 c.c. pipette; azolitmin, 2% solution; filling funnel; pinch cock; sterile test tubes; apparatus for steam sterilization.

Method. **1.** Fresh separated or skimmed milk should be used. Whole milk is undesirable on account of its fat content.

2. Titrate and record the reaction of the milk. If the milk titrates above +1.7% normal, the reaction must be adjusted to +1.5% normal. Sour, curdled or uncurdled milk, after neutralization, does not make a desirable nutrient medium for microorganisms, therefore milk whose titre is above +2 to +2.5% normal should be discarded.

Fresh milk varies in acidity from +1.2 to +1.8% normal. Milk with an acidity above +1.8% normal to phenolphthalein will not give a satisfactory blue color with azolitmin, since at +1.8% normal it begins to show the acid coloration.

3. Add 2% of a standard solution of Kahlbaum's azolitmin. Litmus or azolitmin is added merely as an indicator and should be of sufficient strength so as not to dilute the milk to any extent.

4. Mix the milk and the azolitmin thoroughly and tube, using approximately 8 c.c. of the litmus milk in each tube.

Note. Care should be taken to prevent the milk from coming in contact with the top of the tubes, as it will cause the cotton fibers to adhere to the tube. This may be avoided by the use of a "filling funnel."

5. Sterilize by heating in flowing steam for twenty minutes on four successive days. Milk is difficult to sterilize, owing to the resistant spores which are frequently present. If it is desired to sterilize a larger bulk than in tubes, the time of heating should be lengthened.

Caution: Overheating tends to change (caramelize) the milk sugar. The color of the azolitmin may also be destroyed. These changes are not desirable.

EXERCISE 5. PREPARATION OF POTATO MEDIUM

Apparatus. Large sound potatoes; cylindrical potato knife, or cork borer; ordinary knife; tumbler; sodium carbonate, 1: 500 solution; large sterile test tubes, 1.5 or 2 cm. in diameter and short glass rods, or Roux potato tubes; 5 c.c. pipette; distilled water; apparatus for steam sterilization.

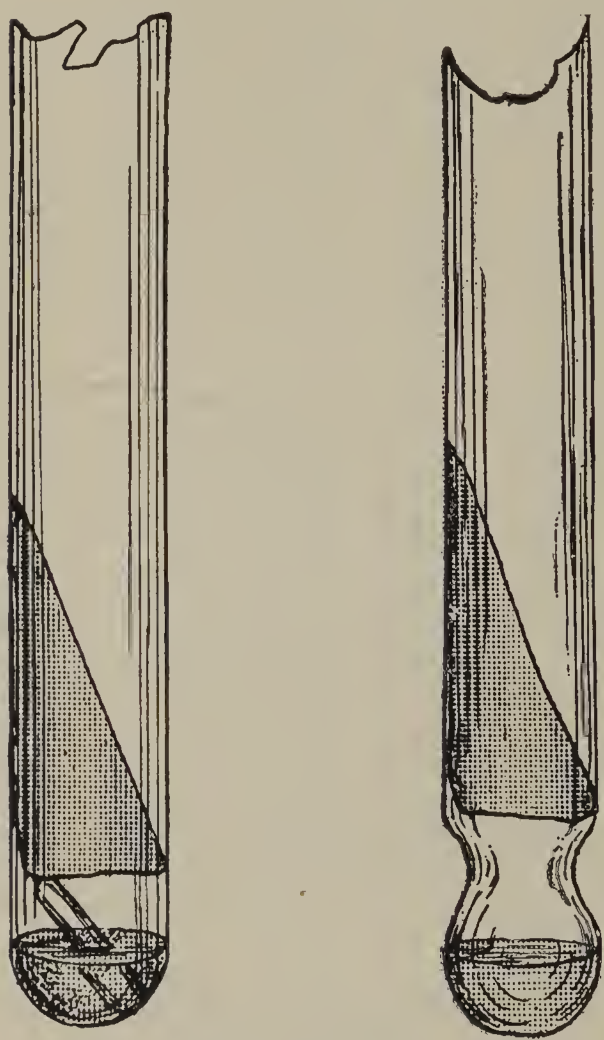


Fig. 8. — Potato Tubes. (Orig. Northrup.)

Method. **1.** Carefully clean one or two large potatoes.

2. By means of a cylindrical potato knife or cork borer, cut cylinders of potato, 4 to 6 cm. long and 1.5 to 1.8 cm. in diameter. With an ordinary knife, halve each cylinder by a diagonal cut so that each piece resembles in shape an agar slant. Remove any portions of the skin on these pieces.

3. Place in a tumbler and soak in a dilute (1: 500) solution of sodium carbonate for thirty minutes. Then wash in several changes of water.

4. Place a glass rod 0.5 cm.×2.5 cm. at the bottom of each tube. Plug with cotton and sterilize. (Roux tubes need only to be cleaned and sterilized.)

5. Place potato slants in sterile tubes, add 3 c.c. of distilled water and sterilize by autoclaving thirty minutes at

fifteen pounds pressure or by heating for twenty minutes in flowing steam on four successive days.

6. Why is sodium carbonate used? Can the same result be accomplished by any other method? To what class of media do potatoes belong?

Note. A number of chromogenic and pathogenic organisms thrive especially well on media containing glycerin. The manner in which glycerin favors the growth of these organisms is not known, but in some instances it seems to be directly utilized for the construction of fat (Bact. tuberculosis). To make a glycerin potato medium, soak the potatoes in a 20% solution of glycerin for thirty minutes after they are removed from the sodium carbonate solution.

REFERENCE

Smirnow, M. R.: The value of glycerated potato as a culture medium. Cent. f. Bakt., II Abt., Vol. 41, p. 303.

EXERCISE 6. PREPARATION OF MEAT INFUSION

Meat infusion and meat extract are the foundations of the ordinary nutrient media, as broth, gelatin and agar, and also of a large number of special nutrient media, as sugar broths, etc.

Some bacteriologists prefer media made from meat infusion, while others prefer to use the meat extract. There are some cases where the one is preferable to the other. When there is a preference, this will be indicated, otherwise meat extract will be used.

Apparatus. 0.5 kilogram (1 lb.) finely chopped fresh lean beef; 500 c.c. tap water; 3.5 liter agateware pail; large funnel; ring stand; clean cheesecloth; one liter measuring cup; sterile liter Erlenmeyer flask; refrigerator; apparatus for steam sterilization (autoclav preferable).

Method. 1. To 0.5 kilogram of finely chopped, fresh lean beef in a 3.5 liter agateware pail, add 500 c.c. of tap

water, mix thoroughly and allow to stand in a cool place (refrigerator preferred) for *not more than* sixteen to twenty-four hours.

2. Set up a large funnel in a ring stand and place a piece of clean cheesecloth in the funnel. Place a clean measuring cup under the funnel.

3. Strain the meat infusion through the clean cheesecloth, thoroughly pressing out all the juice. 0.5 liter should be recovered. If any loss occurs make up to 500 c.c., using tap water.

This resulting sanguineous fluid contains the *soluble albumins* of the meat, the *soluble salts, extractives* and *coloring matter,* chiefly *hemoglobin.*

4. Place the 500 c.c. of meat infusion in a sterile liter Erlenmeyer flask. Replace the plug, and heat in the autoclav at 120° C. for thirty minutes. This is a safer procedure than heating for a longer time in flowing steam.

During this heating the albumins coagulable by heat are precipitated.

It has been found necessary and also more convenient to prepare and sterilize meat infusion before proceeding with the preparation of the different media in which it is used, on account of the resistant spore-forming organisms which are almost universally present in the chopped meat; economy of time also is a consideration. Unless sterilized immediately, meat infusion decomposes quickly owing to the abundance and diversity of the microflora acquired during the various processes of preparation for market.

Infusion made from freshly chopped lean beef will vary in acidity between 1.5% and 2.5% normal. If the reaction is markedly lower or higher, microbial action is taking place, which is, or may be, injurious to the food value of the medium in which the meat infusion is used.

When the infusion has been sterilized and is ready for use, it contains very little albuminous matter and consists chiefly of the *soluble salts of the muscle, certain extractives,* and *altered*

coloring matters along with *slight traces of protein* not coagulated by heat.

EXERCISE 7. PREPARATION OF NUTRIENT BROTH

Nutrient broth is the standard liquid employed for cultivating microorganisms. It is practically a beef tea containing peptone. Peptone, a soluble protein not coagulable by heat, is added to replace the coagulated albuminous substances which precipitate when the meat infusion is sterilized. Salt is added to take the place of the phosphates and carbonates, some of which are precipitated on adjusting the acidity of the medium by sodium hydroxide.

The reaction of ordinary nutrient media is adjusted to about +1.5% normal with phenolphthalein as indicator, as it is found that most microorganisms grow best on a medium neutral or slightly alkaline to litmus.

When it is required that nutrient media be clear, egg albumen reduced to a smooth paste with water (or the well-beaten white of an egg) is added. By coagulation, the egg albumen removes mechanically all small particles in suspension which otherwise would pass through the filter paper. This process is most efficient when the egg albumen coagulates slowly.

As egg albumen begins to coagulate at about 57 C. it is *absolutely imperative* for good results that the medium be cooled to 40°–50° C. before the addition of egg albumen.

Although egg albumen contains small amounts of soluble matter not coagulable by heat, as sugar, extractives and mineral matter, all of which will serve as microbial food, its purpose in nutrient media is primarily for its clarifying action.

Apparatus. 500 c.c. sterile meat infusion; 500 c.c. tap water; 10 gms. peptone, Witte's,* 5 gms. salt; 10 gms. egg

* Where Witte's peptone is specified, any good bacteriological peptone may be used.

albumen (or one egg); 3.5 liter agate-ware pail; titration apparatus; N/20 NaOH; N/1 NaOH; phenolphthalein (indicator); distilled water; 5 c.c. pipette; large stirring rod; coarse balances; large gas burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; sterile 1 liter flask; apparatus for steam sterilization.

Method. **1.** Put the contents of a flask of meat infusion (500 c.c.) in an agate pail and add 500 c.c. of tap water.

2. Add 1% of Witte's peptone and 0.5% of salt.

3. Add 10 gms. of egg albumen which has been well mixed with 100 c.c. of tap water. (Put the egg albumen in a tumbler and add enough water to form a paste. Stir until smooth. Then add the remaining water. One egg* white well beaten may be substituted.) Mix all thoroughly.

4. Heat in autoclav at 120° C. for one hour or longer. This will insure a clear broth unless the reaction is changed considerably. If it is necessary to add large amounts of acid or alkali to adjust the reaction, upon sterilizing the broth, a fine precipitate will appear. Reheating at a slightly higher temperature will sometimes dissolve the precipitate.

5. Titrate with N/20 NaOH.

6. Adjust the reaction of the medium to +1.5% normal with normal NaOH or normal HCl. Retitrate and adjust again if necessary.

7. Pass the filtrate through the same paper till it is bright and clear.

8. Fill thirty sterile test tubes, using approximately 8 c.c. of this medium for each tube. Put the remaining broth in a large, sterile flask.

9. Sterilize the test tubes and remaining broth in autoclav at 120° C. for thirty minutes.

* It is not necessary to add water to the egg.

GELATIN

Gelatin is one of the tools of the microbiologist. As such, it serves two purposes: as a solid culture medium, a technical device by which the isolation of a single species of microorganism is made possible, and, to those organisms which secrete proteolytic enzymes, it serves as a nitrogenous food material.

Gelatin bears the distinction of being the first substance used for a solid culture medium. This medium was originated in 1882 by Robert Koch and has since revolutionized the science of microbiology. Prior to the introduction of solid media, the isolation of a single species of microorganism involved much difficulty and almost always a certain measure of uncertainty. To quote from Jordan: "It cannot be a mere coincidence that the great discoveries in bacteriology followed fast on the heels of this important technical improvement, and it is perhaps not too much to claim that the rise of bacteriology from a congeries of incomplete although important observations into the position of a modern biologic science should be dated from about this period (1882)."

Koch's first plates were made by pouring the liquefied nutrient gelatin upon sterile, flat pieces of glass. The student on becoming familiar with the difficulties of preparing satisfactory plates with the use of the "Petri dish" will appreciate those met with in Koch's first gelatin plates.

Gelatin is a protein, i.e., a nitrogenous food material. It contains as its essential elements carbon, hydrogen, oxygen, and nitrogen (other elements, however, such as sulphur, phosphorus, etc., may be present). Its empirical formula according to Schützenberger and Bourgeois is $C_{76}H_{124}N_{24}O_{29}$, but such a formula only gives information of the chief constituents and allows one to form some idea of the huge size of the molecule; no idea of the structure of the molecule is given. However, by digesting with

dilute sulphuric acid, gelatin breaks down in the same way as the proteins, yielding glycin, leucin and other fatty amino-acids.

Gelatin is an animal protein. but does not occur as gelatin in the animal tissues. It exists there as the albuminoid *collagen* which is the principal solid constituent of fibrous connective tissue, being found also, but in smaller percentage, in cartilage, bone and ligament. Collagen from these various sources is not identical in composition and gelatin varies correspondingly, e.g., gelatin from cartilage differs from that of other sources in that it contains a lower percentage of nitrogen.

Gelatin, the body resulting from the hydrolysis of collagen, is also an albuminoid. (Hofmeister regards this hydrolysis as proceeding according to the equation:

$$C_{102}H_{140}N_{31}O_{38} + H_2O = C_{102}H_{151}N_{31}O_{39}$$

$$\text{collagen} + \text{water} = \text{gelatin}$$

but in dealing with substances of such variable composition, empirical formulæ of this kind have no great significance).

Commercially, it is prepared from certain kinds of bones and parts of skin. These are selected, washed and extracted by water and with a dilute acid (hydrochloric), with relatively little exposure to heat, so that as few as possible of the fluid disintegration products of the stock are formed and the jellying power of the resultant solution is not destroyed.

The term gelatin is derived from the Latin verb *gelare,* to congeal, and calls to mind the principal attribute of this substance, that of its stiffening or jellying property.

Gelatin belongs to that interesting class of substances called colloids. It is a typical example of the class, and exhibits the characteristic properties of the class. Colloids, in marked contrast to crystalloids, do not crystallize, do not readily diffuse and are impermeable to each other. The ultimate particles of colloids are much smaller than

what we would ordinarily term a physical subdivision, but rather larger than chemical molecules; the diameter of the smallest particles in a colloidal solution, e.g., red colloidal gold, which have been counted by means of the ultra-microscope, is 6 millimicrons or 6 thousandths of a micron. A micron is one thousandth of a millimeter. (Bacteria are much larger, the smallest visible by means of the ordinary microscope being from 0.3 to 1.0 micron in diameter.) Consequently their reactions stand midway between the physical and the chemical changes of matter, as may be seen by considering the properties of gelatin.

Gelatin will absorb a considerable quantity of warm water (it is almost insoluble in cold water) and swells up, yielding a jelly which, upon application of heat, melts to a viscous, sticky solution that gelatinizes again upon cooling. The name of *hydrogel* is applied to colloids showing this property. Ordinary gelatin media for microbiological work contain 12% to 15% gelatin. When dried at medium temperatures, gelatin can again be redissolved and redried indefinitely. From this property it is called a *reversible* colloid to distinguish it from other colloids which, when their physical state is once changed, are insoluble, e.g., casein and silicic acid.

If superdried at about 130° C., or superheated when in the gelatinous state either for a short time at a temperature above 100° C., or for a long time at 100° C., as in intermittent sterilization, the gelatin is so modified that its redissolving or resolidifying power respectively is lost. In superdrying, the loss of the redissolving property is laid to the too close contact of the constituent particles, a change in the physical state; in the superheated gelatin, the loss of the resolidifying power is probably due to the disintegration of the gelatin molecule, a more purely chemical phenomenon. This loss of the gelatinizing property is also caused by the enzymic activities of many microorganisms and is also a disintegration process.

Gelatin possesses a liquefaction point which, however, varies considerably under different conditions. Ordinarily, media containing 12% to 15% gelatin will liquefy or melt at a temperature in the vicinity of 24° to 26° C., solidifying again at 8° to 10° C. to a clear, transparent jelly. As a consequence, gelatin media may be employed only for organisms which do not require a higher temperature than 22° to 24° C. for development. Overheating in the process of preparation or sterilization will cause a considerable lowering of the liquefaction point, perhaps ultimately so low that the medium will be liquid at room temperature (20° to 21° C.) It will readily be seen how the latter gelatin medium could not handily be used for the isolation of organisms. A few data will assist in fixing this in mind.

The solidifying property of gelatin varies in inverse proportion with the time of heating during the process of sterilization; *its liquefying point is lowered* on an average of *2° C. for each hour of heating at* 100° *C.* This makes clear why such care must be taken in the preparation of a gelatin medium, in the fractional sterilization of this medium in streaming steam, and why immediate cooling is necessary after each fractionation in the process of its preparation. Although temperatures above 100° C. are much more destructive to the solidifying property than that of 100° C., it is possible to sterilize a medium containing 12% to 15% of gelatin in the autoclav (7 to 8 lbs. pressure) at 112° to 113° C. for twenty minutes or at 15 lbs. pressure (120° C. for five minutes) without impairing its usefulness as a solid culture medium. Both in the lowering of the melting point of agar and gelatin and in breaking down sugars, the time of sterilization is a greater factor than the temperature.

This use of steam under pressure (dry steam) is almost necessary in the case of a gelatin medium to effect sterilization, since gelatin, from its source, method of preparation, and later liabilities to contamination, is almost certain to

contain or bear upon its surface a large number of very resistant spores. Heating at 100° C. for thirty minutes on three or even four or five consecutive days is not always efficient, as these spores do not always germinate within twenty-four hours after heating and, referring to the data above, it is readily seen that the lowering of the liquefaction point is not to be considered as negligible in the process of intermittent sterilization.

Gelatin possesses another property which renders it valuable for bacteriological work: i.e., in gelatin plate cultures no water of condensation ordinarily collects on the cover of the Petri dish (as with agar) later to drop on the surface of the gelatin and thus obliterate forms of colonies and cause isolated colonies to become contaminated with neighboring ones. The storing of this medium either in test tubes or in plates, sterile or inoculated, is thus rendered much more simple than with agar.

REFERENCE

Van der Heide, C. C.: Gelatinöse Lösungen und Verflüssigungspunkt der Nährgelatine, Arch. f. Hyg., Bd. 30, 1897, pp. 82–115.

EXERCISE 8. PREPARATION OF NUTRIENT GELATIN

Apparatus. 3 grams meat extract; 1000 c.c. of tap water; 150 grams gelatin; 10 grams peptone; 10 grams egg albumen; 5 grams salt; water bath; thermometer; 3.5 liter agateware pail; long heavy stirring-rod; titration apparatus N/20 NaOH; N/1 NaOH; phenolphthalein; distilled water; large gas burner; large funnel; plaited filter paper; filling funnel; sterile test tube; sterile 500 c.c. Erlenmeyer flasks; apparatus for steam sterilization; running water bath or refrigerator.

Method. **1.** To 1 liter of tap water add 150 grams of gelatin. Dissolve over water bath. Cool down to 60° C.

2. Mix 10 grams of egg albumen with 100 c.c. of water and add to the dissolved gelatin when it has cooled to 60° C.

3. Now add 3 grams of meat extract, 10 grams of peptone and 5 grams of salt to the above mixture.

4. Cover the pail, place in autoclav and heat at about forty-five minutes at fifteen pounds. Titrate with N/20 NaOH.

5. Adjust the reaction of the medium to +1.5% normal with normal NaOH or normal HCl.

6. Filter while boiling hot through plaited filter paper just previously washed with ½ liter boiling water.

7. Fill thirty sterile test tubes, using approximately 8 c.c. of medium for each tube. Divide the remainder into two equal parts and place in sterile ½ liter Erlenmeyer flasks.

8. Heat in autoclav for twenty minutes at ten pounds pressure.

9. *Cool the gelatin in a running-water bath, immediately.* Care must be taken to heat the gelatin as little as possible, since part of its solidifying power is lost with each application of heat.

AGAR

Agar or agar-agar (from a Malay word meaning " vegetable "), the substance which is used in preparing one kind of solid culture medium for bacteriological work, is a product prepared from various seaweeds found near the Indian Ocean and in Chinese and Japanese waters. This type of seaweed has several common names, as Ceylon or Jaffna moss, Bengal isinglass, etc. Various species are used for food and the trade is considerable.

Payen, a French chemist (about 1859), obtained the agar jelly from the seaweed, *Gelidium corneum,* in the following manner: The seaweed was allowed to stand for some time in a cold dilute solution of hydrochloric acid; the acid was removed by rinsing several times with water, then the seaweed was placed in a cold dilute solution of ammonia;

next the ammonia was removed by repeated rinsing with cold water. During this process, the seaweed lost 53% of its weight in mineral salts, coloring matter, and organic constituents. The remaining portion was boiled in water, during which process the vegetable jelly was extracted. The solution so obtained was poured off, leaving the useless sediment behind. This jelly is the same in composition as that existing in the vegetable tissues; it has not been changed chemically, as is collagen in the preparation of gelatin. The commercial agar is most probably prepared by evaporating this solution to dryness by different means.

Agar usually comes into the hands of the bacteriologist as long, slender, grayish-white strips, or as blocks, or more especially in recent years, in the form of a gray-white powder of European manufacture.

Agar, in contrast with gelatin, is a carbohydrate, i.e., it consists of a combination of carbon, hydrogen and oxygen only. Traces of nitrogen are present as impurities. The above qualitative determinations of its elementary constituents were made by Payen, by Parumbaru and by Hueppe, who made their determinations on agar from different sources. As far as can be ascertained, its empirical formula has not yet been investigated to any extent.

Like gelatin, however, agar is a reversible colloid. It soaks up in cold water, dissolves in hot water after a long boiling to a tasteless and odorless clear solution, and solidifies upon cooling to a more or less opaque jelly. Its watery solution is neutral or nearly neutral to phenolphthalein; still, a drop or two of twentieth normal sodium hydrate is sufficient to make the pink color perceptible.

The colloidal properties of agar are not destroyed by a long-continued heating at a high temperature, nor by the action of ordinary microorganisms as are those of gelatin. The above properties, however, are influenced and may be wholly impaired by the reaction of the liquid in which the agar is dissolved.

The reaction of the liquid, i.e., whether it is acid or alkaline, influences the agar as to its solubility, solidity, color, transparency, filterability and amount of condensation water. If agar is dissolved in a liquid of an acidity equivalent to 0.1% HCl, the agar dissolves very readily, filters quickly, the resultant filtrate being a light yellow, transparent, slippery, watery solution which does not solidify upon cooling. If a smaller percentage of hydrochloric acid is used, solidification occurs (below 40° C.) but the jelly will not " stand up " and is therefore useless for agar slant or plate cultures. A large amount of condensation water is present also.

If agar is dissolved in a weak alkaline or neutral broth, a thick, reddish-brown, viscous liquid is obtained which filters slowly and solidifies quickly at 40° C., to a very solid, opaque, dry jelly, having but little condensation water; it retains its shape well in slants and in plates. Thus the value of the agar as a solid culture medium is raised or lowered according to the degree of alkalinity or acidity.

It must be noted in addition, however, that when once the solidifying property of agar is destroyed by the presence of an excess of acid in its solution, this property can never be regained by neutralization with alkali; the acid permanently destroys the reversibility of the colloid.

The melting-point of agar (of 1.5% in neutral solution) is 97° C. and although its solidifying point is at 40° C., when once it has solidified it will stand up in the thermostat at a temperature of 50° C. For bacteriological purposes, only that form of agar can be used which remains fluid at from 38° to 40° C. Agar which remains fluid only at a temperature above this point would be too hot when in a fluid state for use; the vitality of organisms introduced would be impaired or destroyed by the high temperature.

Difficulties are encountered in the preparation of a solid culture medium from agar, due to its slow solubility, viscosity and consequent slow filterability. Its solution

(digestion) is effected, as mentioned above, by a long heating in a water bath, steam sterilizer, autoclav, or over a free flame. The length of time required for complete digestion depends upon three things: The reaction of the liquid in which the agar is dissolved, the per cent content of agar, and the method of dissolving. The influence of the reaction of agar solutions has been treated above. For general culture use, however, ordinary agar is made +1.5% normal (agar solidifies with difficulty above +3.0% normal).

One per cent agar is much more easily soluble under equal conditions than a higher per cent. One and one-half per cent is the amount used in ordinary agar media, giving a somewhat stiffer and thus more desirable jelly.

Agar is digested most rapidly over a free flame. If not heated sufficiently, after the filtration and sterilization of the agar by the intermittent method, a flocculent precipitate frequently appears in the previously clear medium. This can be made to disappear in most cases by subjecting to the temperature of the autoclav (120° C.—15 lbs.).

Agar for culture media should be entirely clear when liquid, and homogeneously opaque-translucent when solid; it should have a translucence sufficient to allow deep colonies on plates or stab cultures to be observed readily; it should not contain flocculent material, sediment, or pieces of cotton or filter paper, as these hinder typical colony development of microorganisms and, to the inexperienced, may sometimes be mistaken for colonies.

In the first methods ever used for making agar culture media, instead of filtering the hot agar through filter paper, absorbent cotton, or asbestos, it was allowed to cool, during which process the sediment settled to the bottom; when solid the sediment was cut off. This method was not desirable, as the clearness of the resultant agar would depend upon the rate of cooling; the slower the cooling, the more completely would sedimentation take place.

Agar is not a food for microorganisms in general, i.e.,

it is not affected by the digestive enzymes of most bacteria, as is gelatin. However, a few bacteria are known which have the power of liquefying agar, among which are *B. gelaticus* n. sp. (gran) and *Bact. nenckii*, both of which are found, as would be expected, in sea water. This comparative inertness of agar renders it valuable for the preparation of solid synthetic media, the value of which may be enhanced by subjecting the commercial agar to natural fermentation during which process any traces of available food substances are used up by the microorganisms present. (Beijerinck.)

Agar is of special use in bacteriological work in which the cultivation of microorganisms must be conducted at a temperature above the melting-point of gelatin. This feature has made possible the great strides that have been taken in medical bacteriology, as many pathogenic bacteria can be isolated and grown only with difficulty at temperatures below that of the body.

REFERENCES

Smith, Erwin F.: Bacteria in Relation to Plant Diseases. Vol. I, pp. 31–36. Several illustrations.

Schultz, N. K.: Zur Frage von der Bereitung einiger Nährsubstrate. Cent. f. Bakt. I. Orig., Bd. 10, 1891, p. 57.

Von Blom, John L.: California's Seaweed Industry. Sci. Am., Oct. 30, 1920.

EXERCISE 9. PREPARATION OF NUTRIENT AGAR

Apparatus. 3 grams meat extract; 1000 c.c. of a tap water; 15 grams agar; 10 grams peptone; 10 grams egg albumen; 5 grams salt; water bath; thermometer; 3.5 liter agateware pail; long heavy stirring-rod; titration apparatus N/20 NaOH; N/1 NaOH; phenolphthalein; distilled water; large gas burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; sterile 500 c.c.

Erlenmeyer flasks; apparatus for steam sterilization; running water bath or refrigerator.

Method 1. In an agate-ware pail place 15 grams of agar and 500 c.c. of tap water.

2. Wash the agar well, separating the shreds and squeezing through the hands.

3. Decant the dirty water, measuring amount poured off; replace with the same amount of clean tap water. Repeat until clean.

4. Heat the agar over a free flame until dissolved (usually about fifteen minutes, stirring constantly to prevent burning).

5. To 500 c.c. of tap water add 3 grams of a standard meat extract and dissolve (or 500 c.c. of standard meat infusion—see Exercise 6). Then add 1% peptone and 0.5% salt.

6. Mix the latter solution with the agar, cool to 60° C. and add 10 grams of egg albumen mixed in 100 c.c. of water.

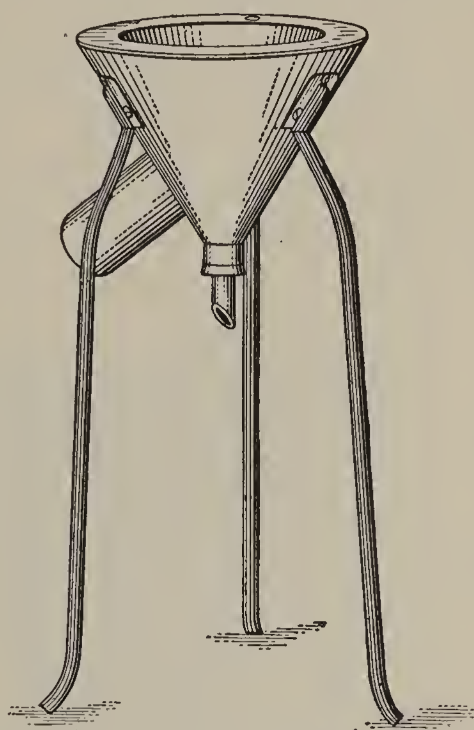


Fig. 9.—Hot Water Funnel for Filtering Agar or Gelatin.

7. Autoclav for two hours at fifteen pounds pressure.

8. Titrate with N/20 NaOH.

9. Adjust medium to desired reaction with N/1 NaOH, or N/1 HCl as the case demands. Retitrate and adjust reaction if necessary.

10. Filter boiling hot through plaited filter paper.

11. Fill 80 tubes, place remainder of agar in flasks and sterilize all of it.

Note. The above method has been very satisfactory in this laboratory. The agar filters very rapidly. If desired, any sugar may be added after filtering.

EXERCISE 10. PREPARATION OF DUNHAM'S PEPTONE SOLUTION

Dunham's solution is utilized for determining the power of microorganisms to produce indol, ammonia or nitrites from peptone, which properties are characteristic of certain species.

Apparatus. 1000 c.c. of tap water; 10 gms. peptone, Witte's; 5 gms. salt; large burner; large funnel; plaited filter paper; filling funnel; sterile test tubes; apparatus for steam sterilization.

Method. 1. Mix 1% peptone and 0.5% salt to a smooth paste with a measured (small) amount of water.

2. Dilute to 1000 c.c. with tap water.

3. Counterpoise and note the weight.

4. Boil ten minutes over a free flame; counterpoise and make up any loss in weight with distilled water.

5. Filter while hot through a plated filter previously washed with hot water. (Filtrate must be perfectly transparent.)

6. Tube, putting 8 c.c. in each tube.

7. Sterilize in the autoclav.

Microorganisms which will not produce ammonia or nitrites from peptone may show this power if nitrogen is added to this solution in the form of inorganic nitrogen as potassium nitrate (0.2%).

EXERCISE 11. NITRATE PEPTONE SOLUTION

This solution is used to determine the power some organisms have of reducing nitrates to nitrites, free ammonia or nitrogen.

Apparatus. 1000 c.c. distilled water; 1 gm. peptone, Witte's; 0.2 gm. nitrite-free potassium nitrate; large agate-ware pail; filling funnel; sterile test tubes; apparatus for steam sterilization.

Method. 1. Mix the following ingredients: 1000 c.c. distilled water; 1 gm. Witte's, or other peptone; 0.2 gm. nitrite-free potassium nitrate. Filter if necessary.

2. Tube, placing 8 c.c. in each tube.

3. Sterilize by heating for fifteen minutes on three successive days or for five minutes in the autoclav at 120° C.

CULTURES

Definitions. A **culture** consists of the active growth of microorganisms in or on a nutrient medium.

A **mixed culture** is a culture composed of two or more species of microorganisms growing together in or on a nutrient medium.

A **pure culture** is the growth of *one* species of microorganism *only*, in or on a nutrient medium, that was sterile before inoculation.

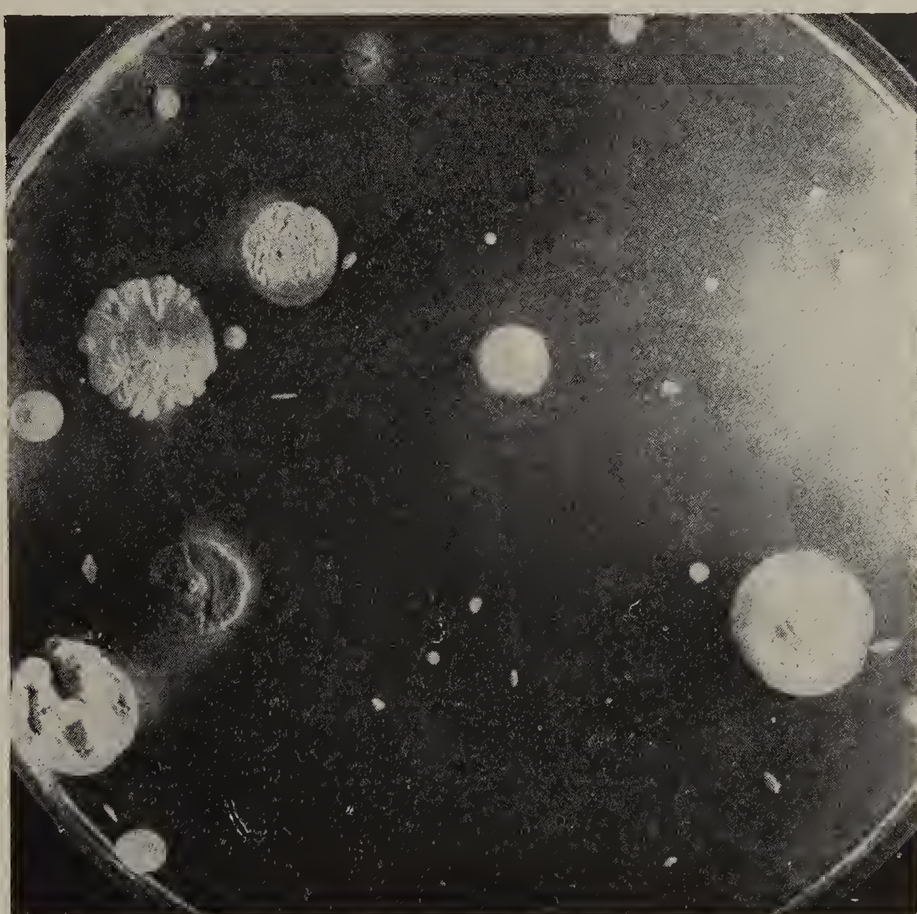


Fig. 10.—Mixed Culture in Petri Dish (Plate Culture) Showing Various Forms and Sizes of Colonies. (Orig. Northrup.)

Pure cultures are used for studying the morphological and physiological characteristics of microorganisms.

From **mixed cultures, pure** cultures may be obtained by the plating method. Mixed cultures of known micro-organisms may be employed in studies on symbiosis, metabiosis, or antibiosis.

Plate cultures are cultures grown in Petri dishes containing a nutrient medium.

Slant cultures is the term generally applied to cultures grown on the inclined surface of any medium, such as agar, potato, blood serum, etc., and are designated specifically as *agar slant cultures, potato slant cultures,* etc. They are generally prepared by drawing a contaminated needle in a straight line along the surface of the medium. Cultures prepared in this way are also frequently termed **streak cultures.** The term streak cultures may also be applied to cultures made similarly but grown on a horizontal flat surface as in a Petri dish.

Fig. 11.—Liquefaction of Gelatin, Saccate becoming Infundibuliform. (Orig. Northrup.)

Slant or streak cultures are valuable in offering a large surface for growth, to aerobic organisms.

Stab (or Stich) culture is the term applied to a culture, generally a pure culture, which is prepared by stabbing a translucent, liquefiable solid medium to a considerable depth with a contaminated straight needle. Gelatin stab cultures are invaluable for studying gelatin liquefaction. Agar is frequently used for stab cultures. If sugar is added to the medium, gas production may be demonstrated. Aerobic and anaerobic bacteria may be easily differentiated by their behavior in stab culture.

Liquid cultures are cultures grown in a liquid medium such as milk, broth, cider, wort, etc.

Shake cultures are made by inoculating with a pure or mixed culture, a liquefied nutrient medium (40°–45° C.). The inoculum is distributed immediately throughout the medium by means of the needle used, or by rotating or shaking.

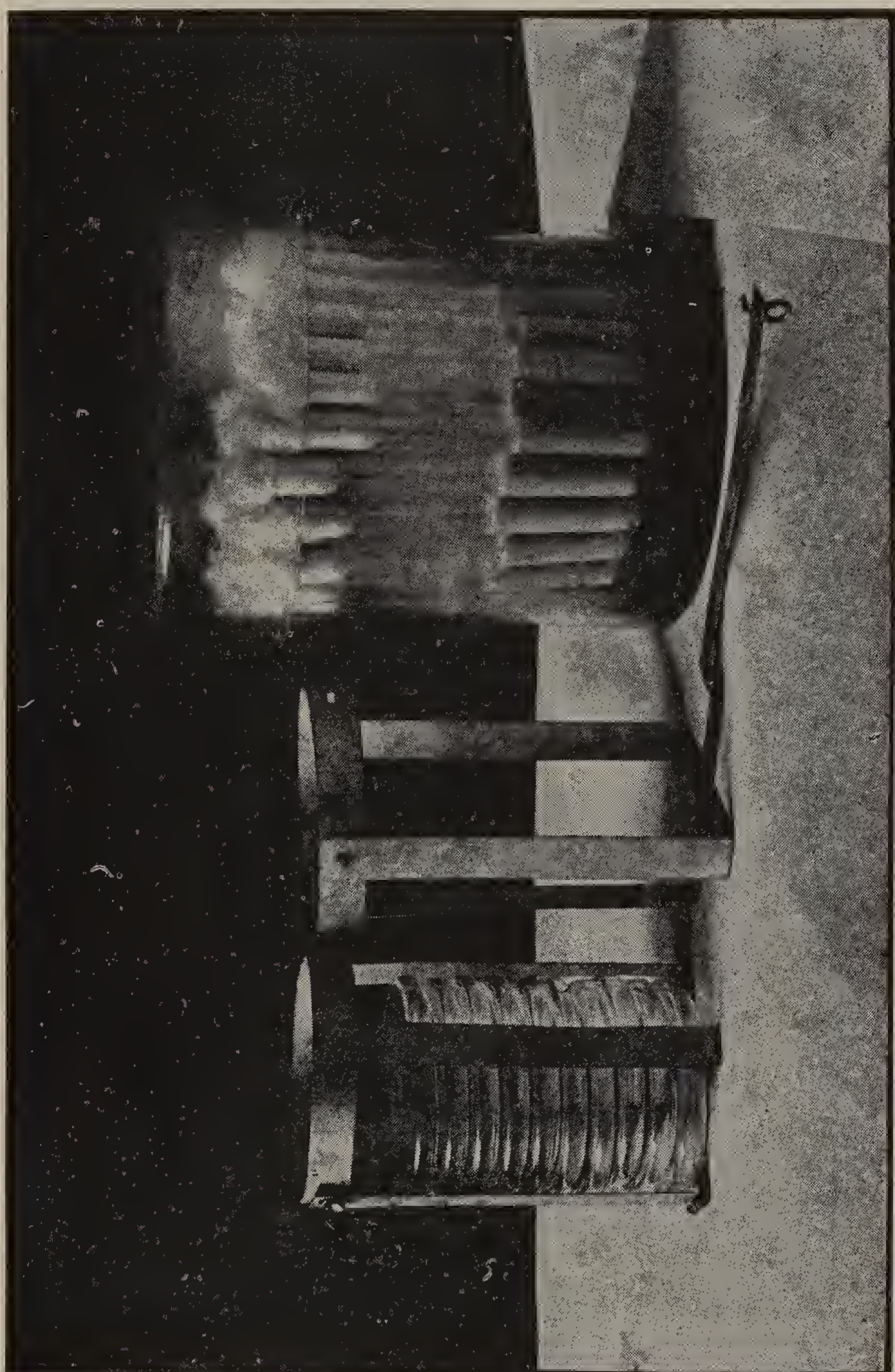


Fig. 12.—Petri Dish Rack and Battery Jar for Storing Media. (Orig.)

This type of cultivation is valuable for determining the oxygen relation of the organisms introduced and is especially useful for demonstrating the presence of gas-producing organisms if a suitable medium is used.

Care of Cultures. 1. *Incubation:* Cultures should be

kept at a constant temperature. Organisms which naturally grow at body temperature (37° C.) as *Bacillus coli*, *Streptococcus pyogenes*, etc., may *with the exception of gelatin cultures*, be kept in the 37° C. incubator.

Always place cultures in tumblers with cotton in the bottom or in small wire baskets; never place them in a horizontal position or incline them carelessly against a vertical surface without proper support.

2. *Care of Broken Cultures.* If a culture of any organism is accidently broken pour 1 : 1000 mercuric chloride, 2% compound solution of cresol or 5% phenol over it and also over any articles which may have been infected; *let stand ten minutes before wiping up.* Always disinfect your hands after handling broken cultures.

3. *Disposal of Old Cultures.* Heat glassware containing cultures to be discarded one hour in flowing steam. Cultures of pathogenic spore-forming organisms should be autoclaved. Glassware so treated may safely be washed by the student.

Never throw living cultures into waste crocks, sinks, or elsewhere. You safeguard yourself and others in the laboratory by destroying all living cultures. *Carelessness in regard to this matter will not be tolerated.*

4. *Care cf Slides, Cover-glasses, etc.* Slides and cover-glasses used for hanging drop mounts, etc., should be immersed in 1 : 1000 mercuric chloride or chromic acid cleaning solution for at least ten minutes before cleaning.

5. *Care of Cuts and Other Wounds.* In case of cuts or wounds, consult the instructor at once. All wounds should be attended to immediately. Tincture of iodin is recommended for painting skin abrasions and deep wounds; in the latter case a bandage should be applied to keep extraneous matter from entering and setting up infection. In case of serious injury, a physician should be consulted. Every laboratory should keep a stock of rolled bandages, etc., for emergencies,

EXERCISE 12. PREPARATION OF PLATE CULTURES, LOOP OR STRAIGHT-NEEDLE DILUTION METHOD (QUALITATIVE)

Plate cultures are a valuable asset to the microbiologist, as they offer a means by which pure cultures of micro-organisms may most easily be obtained; they also allow a quantitative and qualitative study of the microflora of different substances.

Fig. 13.—Water-bath for Melting Agar or Gelatin for Plating, containing a Removable Copper Test Tube Rack. (Orig. Northrup.)

Their preparation consists in, (1) inoculating a liquefied solid culture medium with micro-organisms, (2) mixing them well throughout the medium, (3) pouring the inoculated medium into a sterile Petri dish and, when it has solidified, (4) placing the Petri dish or plate culture at a constant temperature.

The culture medium in solidifying fixes *in situ* the micro-organisms introduced, and well-separated organisms develop into more or less well-separated "colonies" which become visible to the naked eye after twenty-four to forty-eight hours. From these isolated colonies usually pure cultures may then be obtained, or a quantitative or qualitative study may be made.

Isolated surface colonies are most frequently round (concentric in growth) and generally are quite typical for each species, while isolated sub-surface colonies are lenticu-

lar (double concave) or compoundly lenticular in shape as a rule, species differences not being as well defined.

Apparatus. Tripod leveling stand; glass plate about

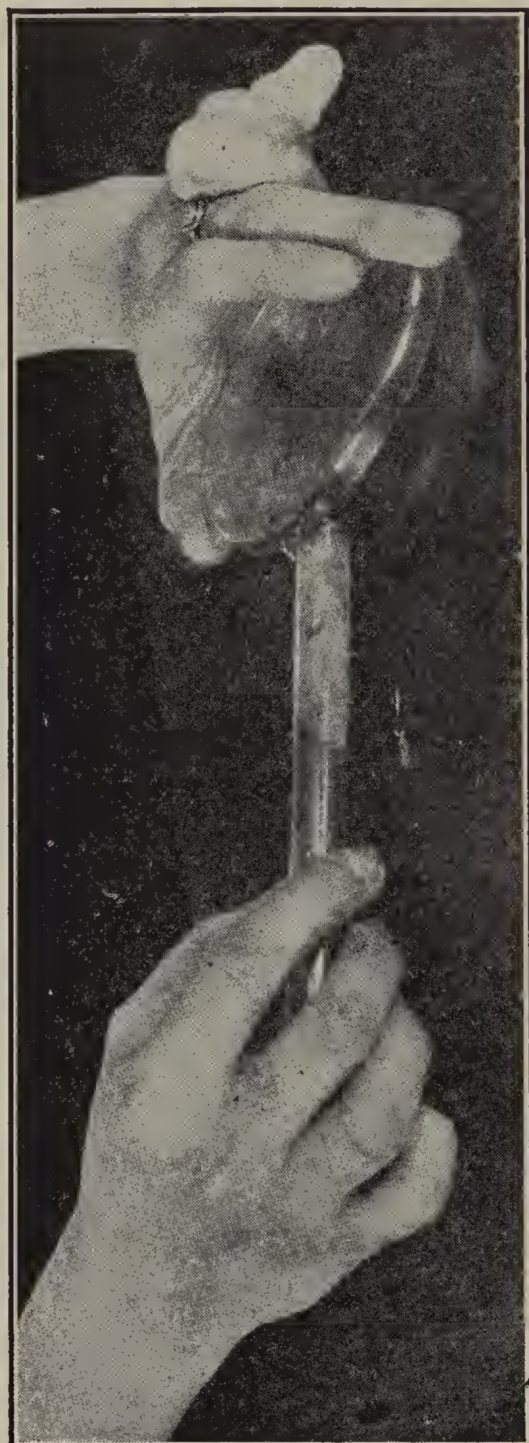


FIG. 14.—Pouring Plate. (Orig. Northrup.)

14 inches square; small spirit level; water-bath; thermometer; sterile Petri dishes; tubes of sterile media (gelatin or agar); culture; platinum needle and loop; Bunsen burner; wax pencil; mixed or pure culture.

I. Procedure for Agar Plates. The loop or straight-needle dilution method is valuable as a quick method of obtaining pure cultures when quantitative results are not desired.

1. Place the glass plate on the leveling stand.

2. Place the spirit level on the glass plate and make level by means of the leveling screws.

Note. The plate-leveling stand facilitates the uniform distribution of the medium over the bottom of the Petri dish, but is not necessary for the accomplishment of favorable results. If the desk top is level this apparatus is unnecessary.

3. Place three sterile Petri dishes, labeled 1, 2 and 3, in a row on the glass plate.

4. Liquefy three tubes of agar at 100° C. in the water-bath or steam and keep at a temperature of 40° to 45° C.

5. Number the tubes of agar 1, 2 and 3 and flame the plugs.

6. With the sterilized platinum needle, merely *touch* the culture and transfer to tube No. 1.

Note. Hold cultures and plugs while transferring as in Fig. 20, p. 57.

7. Distribute the microorganisms through the medium with the needle.

8. Transfer one loopful from tube No. 1 to tube No. 2 and mix with the needle, as in **7**.

9. Slightly raise the cover of Petri dish No. 1. Introduce the flamed mouth of tube No. 1 and pour the melted agar into the plate; remove the mouth of the tube, and replace the cover of the Petri dish. If the medium has not entirely covered the bottom of the plate, tilt slightly in different directions to distribute evenly.

Note. Passing the Petri dish several times through the flame just previous to pouring the plate will aid greatly in preventing the formation of condensation water on the cover.

10. Transfer two loopfuls from tube No. 2 to tube No. 3 and mix.

11. Plate tube No. 2 in Petri dish No. 2 (see **9**).

12. Plate tube No. 3.

13. Label the plates with *name of culture, number of dilution* and *date,* and with your own name or desk number.

14. When the agar has solidified firmly, invert the plates and place in the incubator at 37° C., or at room temperature.

Note. If the plates are placed right side up, condensation water forms on the cover and drops down upon the surface of the agar, causing the colonies to run together and thus destroying their characteristic appearance.

II. Procedure for Gelatin Plates.

1–3. Proceed as in **I.** "Procedure for Agar Plates."

4. Liquefy three tubes of gelatin in the water-bath and keep at a constant temperature of 30° to 35° C.

5–13. Proceed as in **I.** "Procedure for Agar Plates."

14. Place at a constant temperature of 21° C. The gelatin may not harden until placed at this temperature.

Note. Gelatin plates are kept *right* side up, as the organisms may liquefy the gelatin. The liquefied part would then fall from the medium upon the cover and ruin the plate for study.

EXERCISE 13. PREPARATION OF PLATE CULTURES, QUANTITATIVE DILUTION METHOD

In the method given below, "dilution flasks" are prepared containing measured amounts of water or salt solution in which a measured amount of the substance under investigation is placed.

As to whether water or salt solution is used depends upon the nature of the material to be dissolved or placed in suspension. If the substance whose microflora is to be studied contains a certain amount of various salts or other electrolytes in solution, an effort should be made to approx-

imate this amount in the preparation of the diluting fluid, e.g., in obtaining a quantitative estimation of the micro-

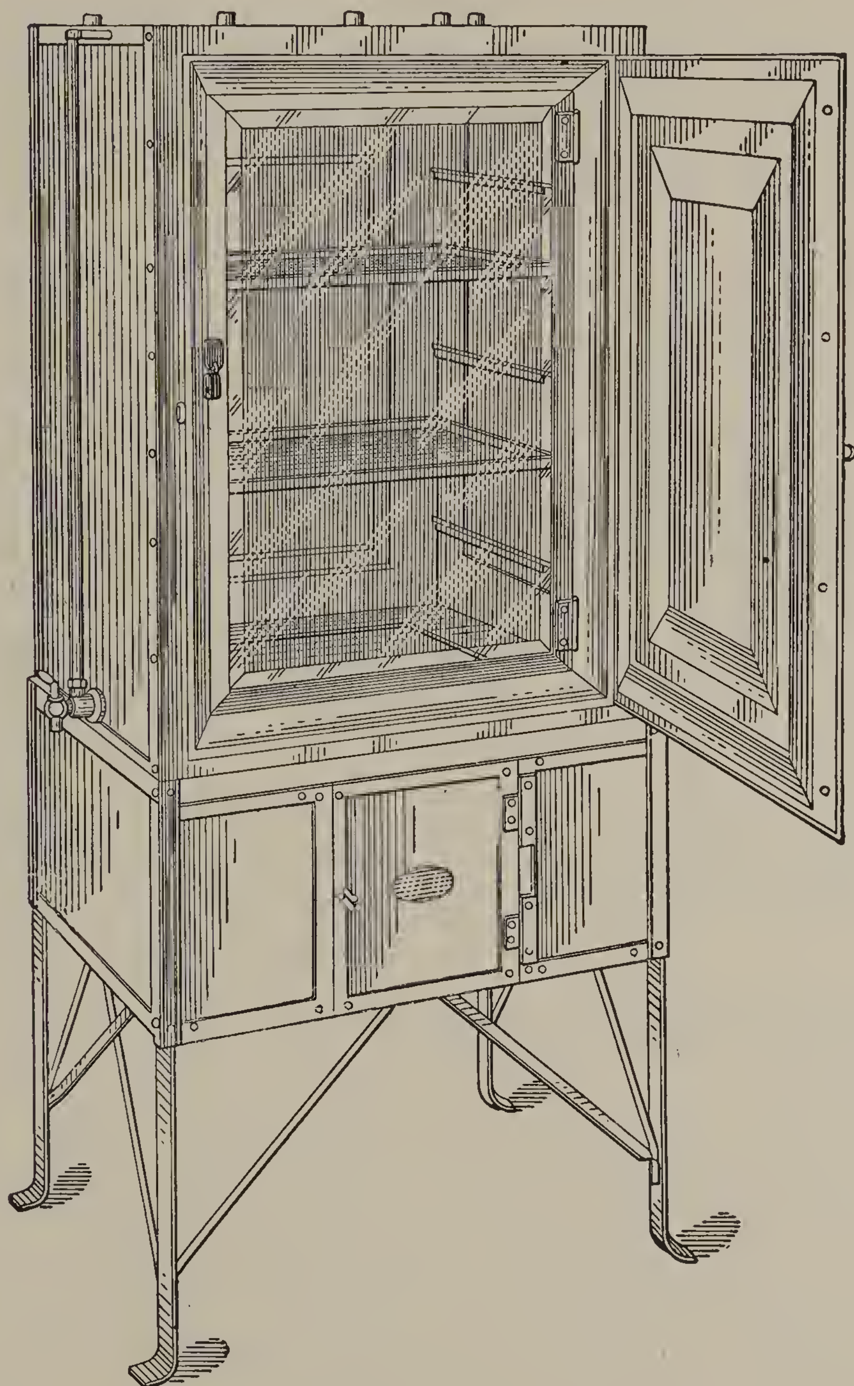


FIG. 15.—Incubator.

organisms from the blood, dilutions should be made in 0.85% salt solution; from tap water, in tap water, etc.

Theoretically, dilutions made in a liquid of a markedly different electrolyte concentration from that of the sub

stance to be studied, might cause either plasmolysis or plasmoptysis as the concentration was respectively too great or too weak.

Microorganisms of different species differ markedly in their susceptibility to osmotic pressure. This cannot be determined, however, unless studies are made of pure cultures of each, therefore the percentage of salt in the diluting liquid should approximate that of the substance whose microflora is to be studied.

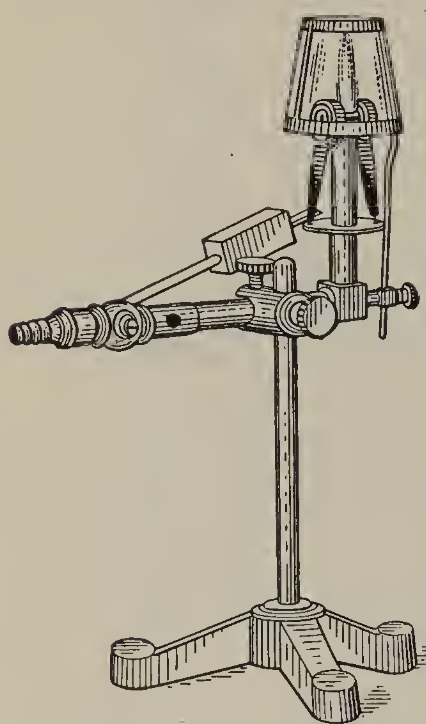


Fig. 16. — Koch's Safety Burner for Incubator or Water Bath.

The method below is applicable to substances in the liquid condition only. Modifications of this method may be utilized to apply to nearly every class of substances.

Plates are generally made from *three* different dilutions, so that well-separated colonies may be obtained on at least two plates.

Apparatus. Sterile 1 c.c. pipettes (graduated to 0.1 c.c.); sterile 10 c.c. pipettes (graduated); sterile Erlenmeyer flasks of 200 c.c. capacity containing 90 c.c. and 99 c.c. of sterile water or salt solution; three sterile Petri dishes; three tubes of sterile agar or gelatin.

Note. Use only freshly prepared dilution flasks, otherwise evaporation takes place so rapidly that accuracy is not possible.

Culture. Substance under investigation.

Method. 1. With a sterile 1 c.c. pipette, transfer 1 c.c. of the original sample or culture to a flask containing 99 c.c. of sterile water or salt solution. The flask now contains 100 c.c. of liquid containing 1 c.c. of the original sample, giving a dilution of 1 in 100.

Note. A sterile pipette must be used for each separate operation.

2. Shake the flask to secure an even suspension of the microorganisms.

Do not allow the liquid to touch the cotton plug.

3. With a sterile pipette, transfer 1 c.c. of the first dilution into a flask containing 99 c.c. of sterile water and shake. The second flask now contains 100 c.c. of a liquid containing 1/100 of the original sample, a dilution of 1/100 in 100, or 1 in 10,000.

4. If a higher dilution is required, 1 c.c. from the flask containing the 1/10,000 dilution placed in a flask containing 99 c.c. sterile water gives 100 c.c. of a liquid containing 1/10,000 of the original sample, or a dilution of 1 in 1,000,000.

If a lower dilution of the original sample than 1/100 is desired, make use of the 90 c.c. dilution flasks as follows:

With a sterile 10 c.c. pipette place 10 c.c. of the original sample into 90 c.c. of sterile water and shake. This flask now contains 100 c.c. of liquid containing 10 c.c. of the original sample, giving a dilution of 1 in 10. A dilution of 1 in 1000 may be made either by placing 1 c.c. of the 1/10 dilution in 99 c.c. of sterile water, or by placing 10 c.c. of the 1/100 dilution in 90 c.c. of sterile water.

Note. Almost any desired dilution can be made by the use of these flasks.

5. *For plating*, transfer 1 c.c. with a sterile 1 c.c. pipette from the flask containing the desired dilution to a sterile Petri dish.

Note. *Never use less than* 1 *c.c.* Run duplicates when greater accuracy is necessary.

6. Liquefy the desired number of agar or gelatin tubes in the water-bath or steam at 100° C.

7. Cool to a temperature of 40° to 45° C.

8. Pour the plates, tilting each carefully so that the 1 c.c. of the diluted sample may be mixed well throughout the medium.

9. Place the plates on a level surface until the medium solidifies.

10. Incubate at the desired temperature.

EXERCISE 14. METHODS OF COUNTING COLONIES IN PETRI DISH CULTURES

Apparatus. Jeffer's counting plate; black glass plate or cardboard; tripod counting lens, magnifying four diameters; plate cultures.

Note. In Jeffer's counting plate (see illustration) each division has an area of 1 square centimeter. The figures denote the number of square centimeters in the respective circles.

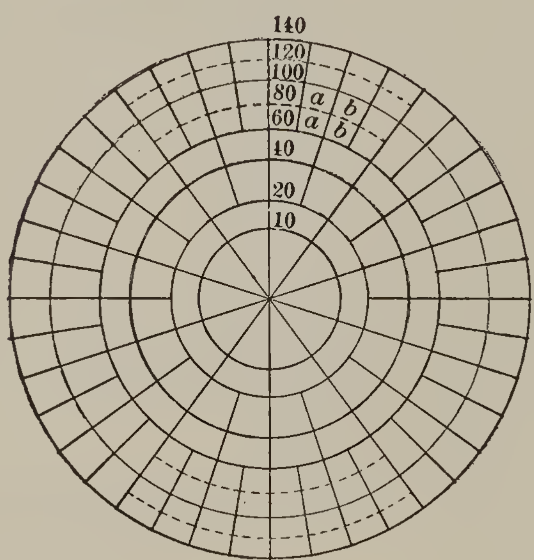


Fig. 17.—Jeffer's Counting Plate.

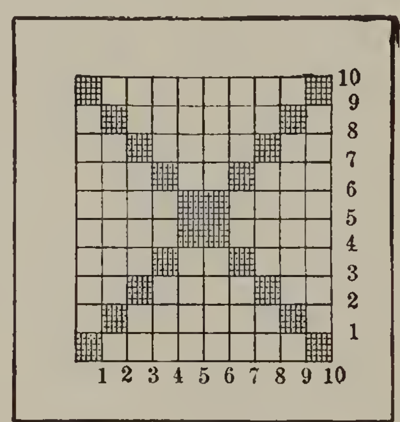


Fig. 18.—Wolfhügel's Counting Plate.

Method. **1.** Invert the Petri dish culture to be counted upon the black glass plate or upon some black surface.

Note. If liquefiers are present on the gelatin plate, place the Petri dish right side up upon the counting plate; this necessitates refocusing the lens. The cover may be removed to facilitate counting if the plate is to be discarded.

2. Place the counting plate upon the Petri dish, making the circumference of the Petri dish coincide as nearly as

possible with that of one of the circles on the counting plate.

3. Using the tripod lens count the colonies in each sector of the smallest circle, then in each division between the concentric circles.

Note 1. The tripod counting lens must be used if the colonies are very small, as they otherwise may be confused with air bubbles in the medium. If there are less than 500 colonies present, the entire plate should be counted. If the number is much greater, from ten to twenty divisions, in some definite order, should be counted, an average taken, and the results multiplied by the area of the plate in square centimeters.

Note 2. Wolfhügel's counting plate is very desirable for counting a large number of colonies. It is ruled in square centimeters and the squares on the diagonals of the plates are subdivided into smaller squares. The colonies appearing in from ten to twenty of these smaller squares may be counted, an average taken and the result multiplied by the number of small squares in 1 sq. cm. times the area of the Petri dish in square centimeters. (The entire area of the plate may be obtained most quickly by placing a Jeffer plate upon the Petri dish in question.)

4. Ascertain the number of colonies per cubic centimeter in the original sample by multiplying the whole number of colonies on the plate by the dilution; e.g., if there are 386 colonies on the plate and the original culture was diluted 1 in 1000, the number of colonies contained in each cubic centimeter of the original sample is 386,000.

Note. When there is an excessive number of colonies on a plate the vigorous microorganisms will inhibit the growth of the less vigorous and thus the number of colonies counted is smaller than the number of microorganisms present. Moreover, the colonies may become confluent and the counts will again be in error. From 50 to 200 colonies on a plate is considered a desirable number.

EXERCISE 15. ISOLATION OF MICROORGANISMS FROM PLATE CULTURES AND METHOD OF MAKING AGAR STREAK CULTURE

Apparatus. Straight platinum needle; several tubes of sterile agar slants; Bunsen burner; wax pencil; plate containing from 30 to 200 well-separated colonies.

Method. **1.** Examine the plate to determine the colonies which differ macroscopically and microscopically. (Use

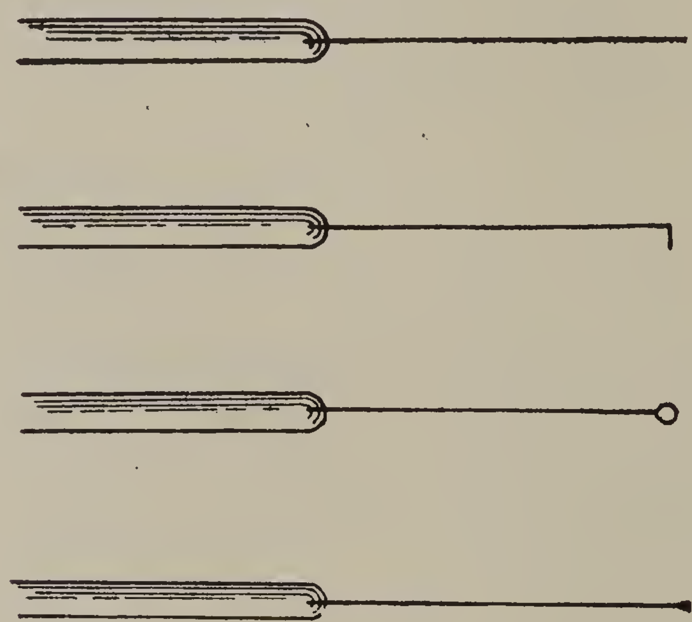


FIG. 19.—Various Forms of Platinum Needles. (Orig. Northrup.)

a counting lens or the lowest power of a compound microscope.)

2. Note the most isolated of each kind, and mark them with the wax pencil upon the bottom of the plate to insure picking up the proper colonies later. Also note how the deep and surface colonies differ.

3. Examine each marked colony under the lowest power of the microscope to make sure of its purity. If the colony does not appear to be wholly isolated, pick up a small portion of it with a sterile platinum needle and stain with one of the common stains (see Ex. 28) or examine it in the

hanging drop (see Ex. 21) to determine if more than one kind of organism is present.

4. If the colony is pure, pick up a portion with the

Fig. 20.—Transferring Cultures. (Orig. Northrup.)

sterile needle, or, in case of extremely small colonies, remove the cover from the plate, focus the low power of the microscope on the desired colony and while looking through the microscope, fish the colony.

5. Transfer to one of the agar slants, making a streak along the median line of the inclined surface of the agar, drawing the needle from the base to the top of the slant.

Note. For investigational purposes, when dealing with unknown microorganisms, the following method is more accurate for obtaining them in pure culture: Transfer to a tube of broth; incubate for twenty-four hours and plate a second time. Isolate from the twenty-four-hour plate culture.

6. Incubate at the optimum temperature.

Note. If the agar slants have become dried out to any extent, it is necessary that the agar be melted and re-slanted in order that optimum growth may take place.

EXERCISE 16. METHOD OF MAKING TRANSFERS OF PURE CULTURES INTO A LIQUID MEDIUM

Directions for making transfers of pure cultures from one medium to another must be followed *very carefully*, otherwise extraneous microorganisms may enter and hopeless confusion result.

Apparatus. Test tubes containing a sterile liquid nutrient medium; platinum needle; Bunsen burner.

Culture. Pure culture.

Method. 1. Flame the cotton plugs of the test tubes containing the pure culture and the sterile liquid nutrient medium.

2. Sterilize the platinum needle in the flame.

3. Permit it to cool (about one minute is required).

4. Hold it in the right hand and remove the cotton plug of the culture tube with the little finger of the same hand.

5. Take up a very little of the culture with the needle.

6. Replace the plug of the culture tube.

7. Remove the plug of the tube of sterile liquid medium in the same manner.

8. Insert the infected needle into the liquid.

9. Replace the plug.

10. Sterilize the needle before laying it down.

EXERCISE 17. METHOD OF MAKING STAB CULTURES

Apparatus. Tubes of sterile agar or gelatin; straight platinum needle; Bunsen burner.

Culture. Pure culture.

Method. 1. Liquefy the gelatin or agar tube and resolidify it in a vertical position in cold running water or in some cold place.

2. With a sterilized *straight* platinum needle pick up a very little of the culture or colony.

3. Insert the needle at the middle of the circle made by the surface of the medium and push the needle about 5 cms. into the solid medium (within 1 cm. of the bottom of the tube), then withdraw carefully so that the path of the needle be as limited as possible. The microorganisms grow along the path of the needle.

Avoid having the shoulder of the rod come in contact with the surface of the medium lest its heat disfigure the surface or even kill the microorganisms. The surface of the medium should remain intact during this process.

4. Replace the plug in the new culture and sterilize the needle.

EXERCISE 18. PREPARATION OF A GIANT COLONY

Purpose. To show the development of a single colony of a microorganism.

Apparatus. Sterile Roux culture flask* or Petri dish; tubes of agar or gelatin.

Culture. Organism to be studied.

Method. 1. Melt two tubes of dextrose agar or gelatin. Pour into the culture flask.

Note. Allow the medium to touch and cover one large side *only.*

2. Heat in this horizontal position in flowing steam fifteen minutes.

3. Distribute the medium evenly over the large side,

* Most valuable for molds, especially *Rhizopus nigricans.*

and set on a level surface to cool. When the medium has solidified, place the flask with the medium-side up.

4. Mark the center of the flask on the outside with a

Fig. 21.—Giant Colony of Mold in Roux Flask. (Orig.)

wax pencil and inoculate with a bent platinum needle in one spot *only*.

Note. When making mold inoculations *moisten the sterile needle with sterile water or medium before touching the spores;* this insures a positive inoculation.

5. Keep at room temperature medium-side up.

6. Examine and measure the diameter of the giant colony from day to day and describe the typical growth

of the colony, using the terms on the descriptive chart of the Society of American Bacteriologists, p. 134, as far as possible.

7. Compare the giant colonies of a *Mucor*, *Pencillium*, a yeast and *Bacillus subtilis* or *Bacillus mycoides.* Use agar for giant colonies of these bacteria, as they liquefy gelatin.

8. Giant colonies of yeasts and bacteria and some molds may be grown in Petri dishes, or in flat-bottomed flasks.

For illustrations of giant colonies of bacteria see:

FUHRMAN: Vorlesungen über Technische Mykologie, pp. 41, 43.

LÖHNIS: Vorlesungen über Landwirtschaftliche Bakteriologie, pp. 38, 170.

LEHMANN AND NEUMANN: Bakteriologie und Bakteriologische Diagnostik, Bd. I. (Atlas.)

For illustrations of giant colonies of yeasts see:

LAFAR: Technische Mykologie, Bd. 4, German Ed., pp. 24–25, 306, and above references.

THE MICROSCOPE

Care of the Microscope. For microbiological work a compound microscope is necessary. This should be fitted with a minimum of two oculars corresponding to the Leitz No. 1 (lowest power) (Spencer, 6×), and No. 3 or 4 (Spencer, 10×) and three objectives corresponding to the Leitz $\frac{1}{3}$ in. (lowest power) (Spencer, 16 mm.), $\frac{1}{7}$ in. (Spencer, 4 mm.) objectives (dry) and $\frac{1}{12}$ in. oil immersion objective. A coarse and a fine adjustment permit the accurate focusing of any combination of lenses. The substage should be fitted with a good condenser and iris diaphragm for regulating the amount of light, and a plane-concave mirror.

Great care should be exercised in the use and care of the microscope as it is a delicately adjusted instrument.

The following rules should be heeded:

The Stand. The stand is the body of the microscope carrying the optical parts.

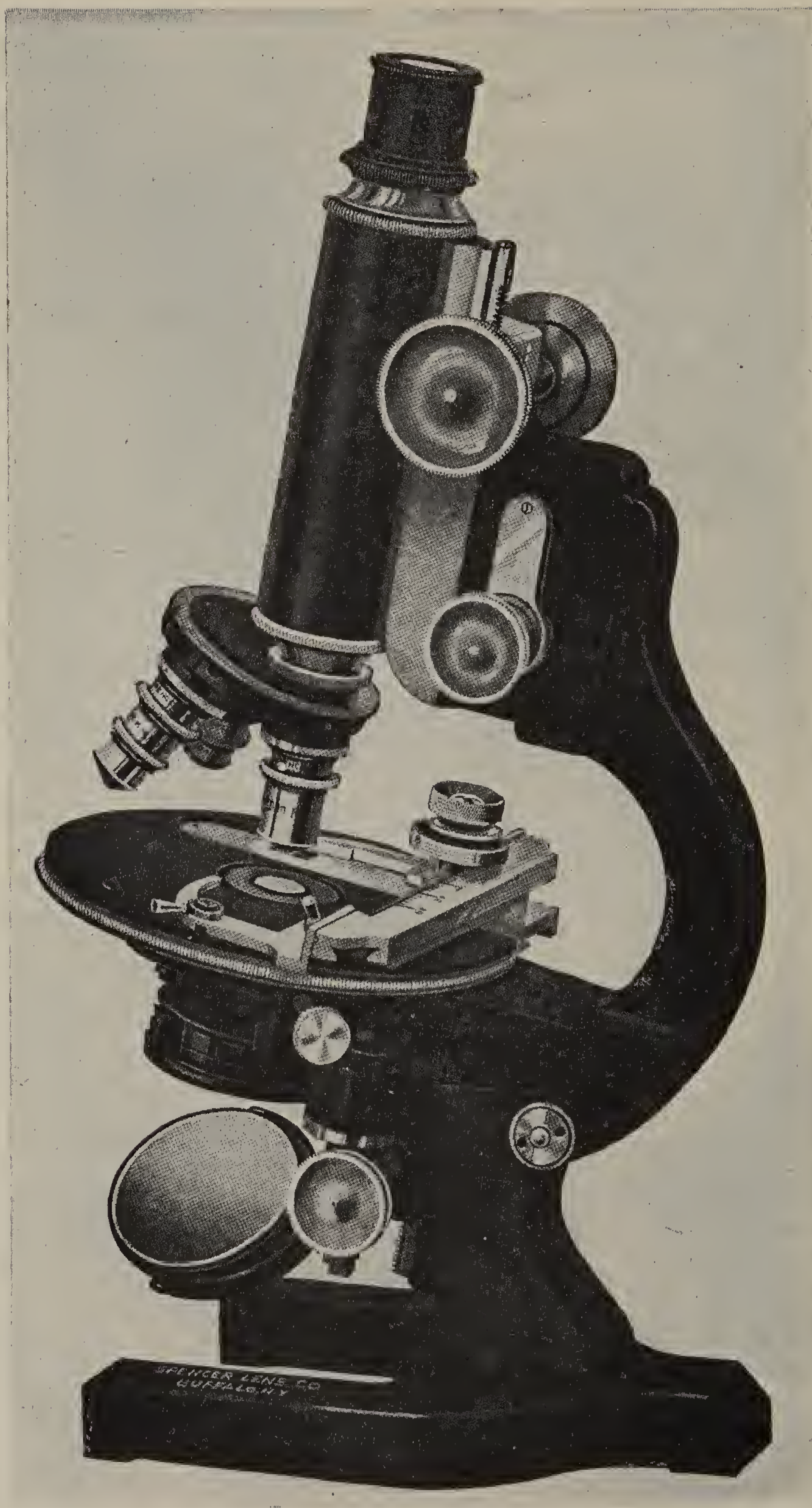


FIG. 22.—Compound Microscope with Mechanical Stage Attached and Side Fine Adjustment.

1. *Leave the microscope in the case when not in use.* Dust works into the bearings of the instrument, making them work hard and unnecessarily wearing them.

2. When handling the microscope do not grasp it by the arm which contains the fine adjustment unless the microscope is designed to permit this. Grasp it by the pillar below the stage.

3. *Never use alcohol on the lacquered parts.* Rubbing gently with a very little xylol and drying quickly will remove any oily material.

The Stage. The stage is that portion of the microscope on which the mounted object is placed for examination.

1. Should the stage become soiled with balsam, immersion oil or anything which water will not remove, it can be cleaned with xylol or chloroform. A little heavy oil will restore the stage to its original black color.

The Fine Adjustment. The fine adjustment is used for bringing out details in very small objects and is necessarily of limited range and delicate in its mechanism.

1. If, when looking into the eye-piece, no change of focus is noticed by turning the micrometer head, or if the micrometer head ceases to turn, the adjustment has reached its limit. To adjust, focus down or up, respectively, with the coarse adjustment, and turn the micrometer head until the fine adjustment is midway within its range.

2. When the fine adjustment screw stops, *do not force it.*

The Draw Tube. The draw tube is the tube receiving the ocular.

1. The draw tube should work easily and smoothly. On the draw tube will be found graduations in millimeters or inches, some fixed point at which certain combinations of objectives and oculars give the clearest image. This differs with different microscopes and should be known for the microscope used.

The Nose-piece. The triple nose-piece on the compound microscope serves a double purpose; to obviate the neces-

sity of screwing the different objectives in as needed and to protect the back lens of the objective from dust. The later microscopes have a "collar" nose-piece which keeps the objectives free from dust at all times.

1. Nose-pieces and objectives of the best makes are now made so that the objectives are *parfocal*, i.e., when one lens is in focus the others on the nose-piece will be nearly in focus when they are swung into the optical axis. They are also approximately centered so that a point in the center of the field of one lens will be in the field of the others.

2. Objectives made parfocal for one tube-length or eye-piece are not parfocal for a different length or a different eye-piece.

3. Objectives of one microscope should not be interchanged with those of another, even if of the same make.

4. *Always focus up*, slightly, before turning from a lower to a higher power. Otherwise the front of the objective may be swung against the cover-glass and injure both the specimen and the objective.

The Optical Parts. The optical parts are the lenses of the objectives, oculars and condenser and the mirror.

1. Wipe dirty lenses gently with Japanese lens paper to remove dirt.

2. Never rub a lens vigorously with anything.

3. Avoid touching the surface of a lens with the fingers. Cutaneous secretions are hard to remove.

4. Always clean the oil immersion objective with lens paper *immediately after using*. If the oil is allowed to dry, xylol must be used to clean the lens.

5. *Always leave an ocular in the tube* to keep dust from settling on the back lens of the objective. Dust on the back lens may be removed with a camel's hair brush.

6. *Never take an objective apart.*

7. Oculars, condenser and mirror should be kept clean by the use of lens paper.

Use of the Microscope. Position. 1. Always use the microscope with the tube in the perpendicular position. This is indispensable in examining fresh mounts or fluids.

2. *Work with both eyes open* and if possible use both eyes interchangeably.

Light. 3. *Never use direct sunlight.* The best light is obtained from white clouds. Northern or eastern light is preferable.

The best artificial light is a Welsbach burner (gas). When employing artificial light use a blue glass between

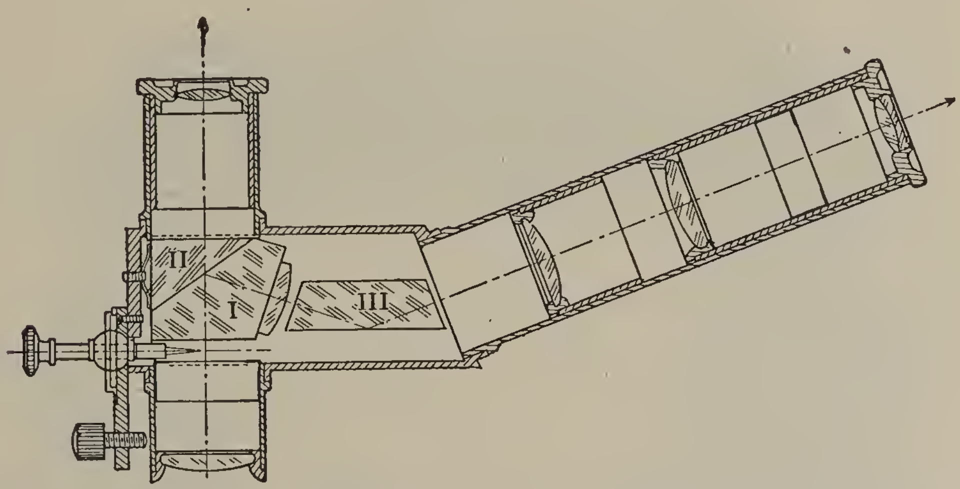


Fig. 23.—Double Demonstration Ocular with Pointer Enabling Two Observers to View Simultaneously the Image Indicated by the Adjustable Pointer.

the light source and the specimen. Often an eye-shade or some appliance with a similar purpose is desirable.

4. Use the plane mirror in daylight, the concave mirror with artificial light.

Focusing. 5. After putting in place a low-power ocular and objective, place the specimen on the stage, and while looking through the microscope, adjust the mirror so as to illuminate the field as evenly as possible, but not so brightly as to irritate the eyes.

6. By means of the coarse adjustment, focus the body tube until the objective *nearly* touches the cover-glass, *being careful not to touch it.*

7. With the eye at the ocular, focus up slowly with the coarse adjustment until the specimen comes plainly into view.

If the light is too intense the focal point may be passed without noticing it.

8. When the object is brought fairly well into focus by

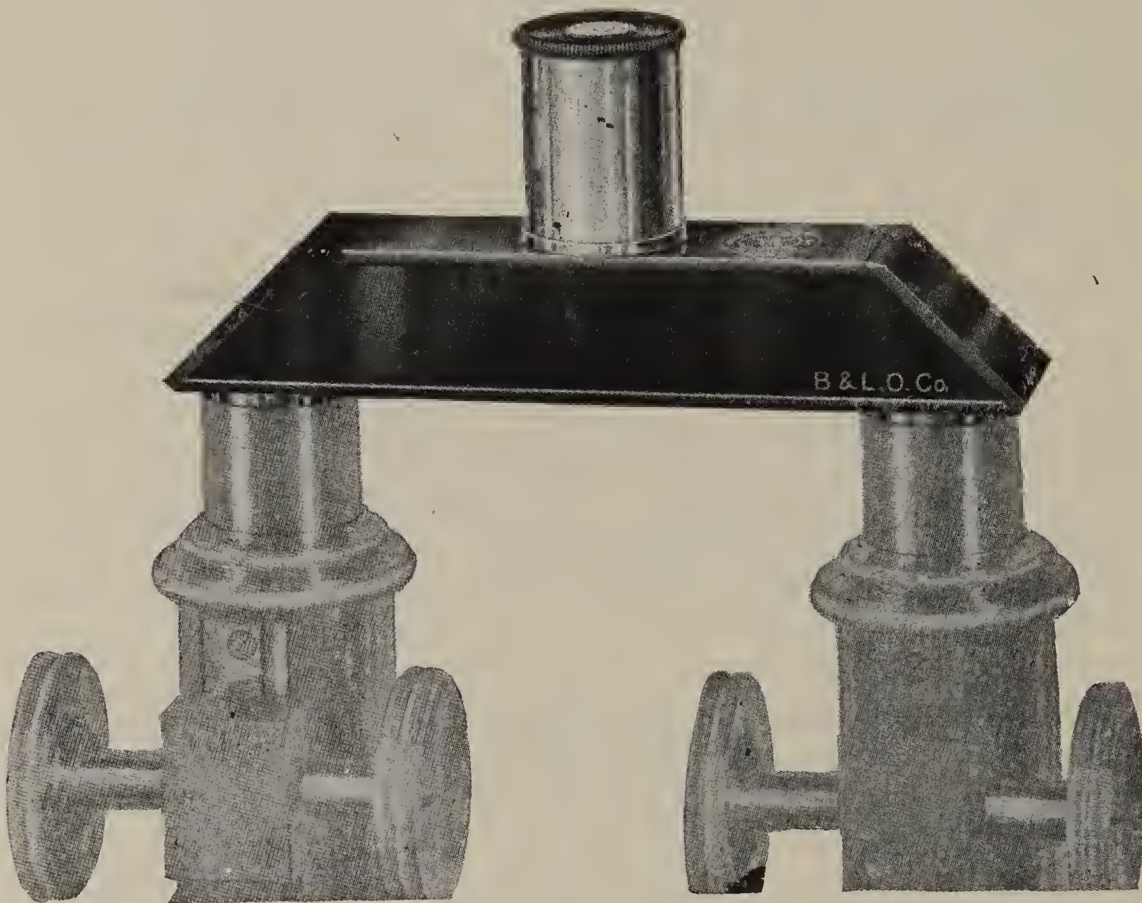


Fig. 24.—Comparison Ocular Enabling One to Observe Two Different Microscopic Fields Side by Side. Any two microscopes may be used. (Healy.)

means of the coarse adjustment, use the fine adjustment to obtain the sharpest focus to bring out details.

9. Move the specimen when trying to obtain a focus, as a moving object is more apt to be noticed as the lens comes into focus.

The microscope *reverses* the image. This will be noticed when the specimen is moved. The microscope magnifies the movement as well as the image; it therefore requires

a certain delicacy of movement to put a specimen in a desired position.

10. *Beginners should always use the low-power objectives and oculars first.* The low-power objectives have longer working distances and always show a larger portion of the specimen. After obtaining a general idea of the specimen, desired portions may be examined with the higher power objectives.

11. In using high-power objectives for finding and examining a specimen, it is always more desirable to use the lowest power ocular (corresponding to Leitz No. 1). If a higher ocular is used, there is a loss in the depth or sharpness and size of field, since they are both inversely proportional to the magnification. Illumination is also lost, which varies inversely as the square of the magnification. Remember that *the largest field, the greatest penetration, and the best illumination are obtained by using the lowest magnification which makes all the detail in the image visible.*

Oil Immersion Objective. The highest power objective is the oil immersion lens. This is so termed because a drop of oil must be used between the front lens and the cover-glass. The oil used must have the same index of refraction as glass to prevent the dispersion of the rays of light coming from the condenser.

Working distance is the free distance between the cover-glass and the objective when the latter is focused. High-power objectives have short working distances.

REFERENCE

Gage: The Microscope.

EXERCISE 19. METHOD OF MEASURING MICRO-ORGANISMS

I. Using the Leitz Ocular "Step" Micrometer. In this ocular micrometer the intervals are arranged in groups of ten, each group being indicated by black steps rising from the first to the tenth interval.

This arrangement possesses the great advantage that the divisions can always be seen distinctly whether the objects be light or comparatively dark.

The intervals of the scale, instead of being 0.1 mm. or 0.5 mm. wide, as in ordinary ocular micrometers, have a definite value of 0.06 mm. This gives for each objective and for a given tube length, convenient and in many cases integral micrometer values, which renders a greater facility in the use of this instrument. The actual tube length differs in most cases but little from the standard length. The tube length and the micrometer value of each microscope, however, should be separately calibrated.

It is of importance to be able to determine the size of microorganisms: (1) because it is of general interest to know the size of the microorganisms with which we are dealing; (2) because the difference in size is an important factor in identifying and describing the organism; (3) because the size is necessary for purposes of comparison with other microorganisms.

Apparatus. Microscope; Leitz ocular "step" micrometer; object micrometer; specimen to be measured.

Method. **1.** With the aid of the Leitz ocular "step" micrometer the size of stained or unstained microorganisms on either a light or a dark field may be measured directly in microns.

A **micron** is 0.001 mm., and is expressed by the Greek letter μ.

2. One hundred divisions of the step micrometer cover 100, 15 and 10 divisions of the object micrometer

when Leitz objectives 3, 7 and 1/12 oil immersion are used.

The object micrometer is simply a cover-glass (mounted on a slide in Canada balsam) upon whose surface has been ruled a scale 2 mm. in length, each millimeter being divided into 100 equal parts, the space between each division therefore being equal to 0.01 mm.

3. If 100 division lines of the ocular step micrometer cover 0.01 mm. of the object micrometer, then each division line of the step micrometer has the value 0.001 mm. or 1.0 micron.

These values are only accurate when the draw-tube of the microscope is drawn out according to the following table.

4. *Using the ocular step micrometer and the object micrometer, find the tube length at which each objective gives a definite value in microns.* This will vary some even with the Leitz oculars and objectives, so the tube length for each combination of lenses must be determined separately for any make of microscope.

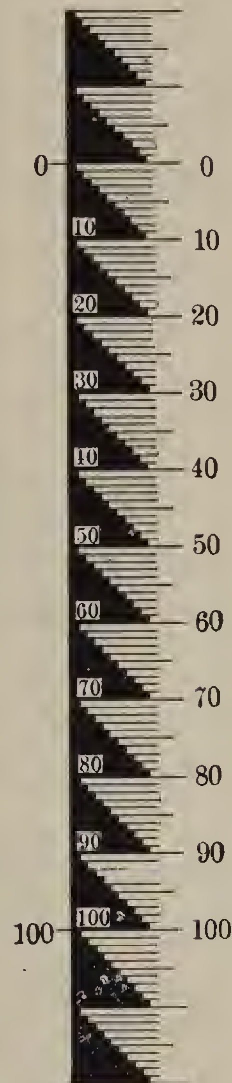


Fig. 25.—Micrometer Scale in Ocular of the Leitz Ocular Step Micrometer.

VALUES FOR LEITZ ACHROMATIC OBJECTIVES

No. of Objective.	Mark on Draw-tube.	Micrometer Value in Microns of Each Division Line of Step Micrometer.
3	141	10
7	174	1.5
1/12 oil imm.	150	1.0

5. *Multiply the number of division lines of the ocular micrometer covered by the organism in question by the value*

of the division line as determined in the above table. This gives the measurement directly in microns.

Microorganisms may be measured more accurately by mounting them in Chinese ink, as they cannot move, are not shrunken or distorted as often occurs with stained specimens, and are clearly seen. Preparations stained with

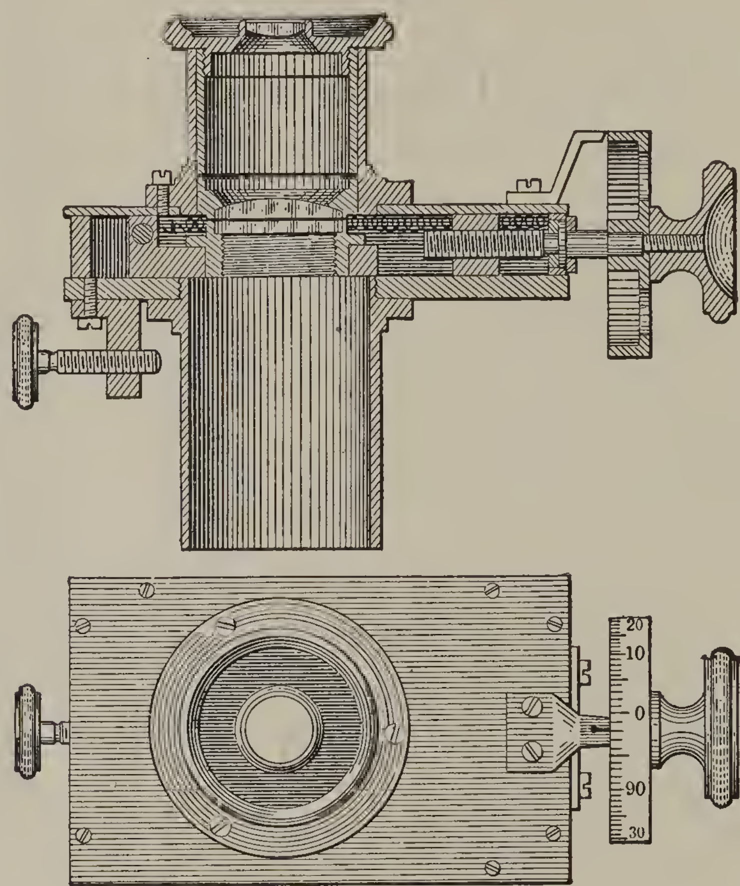


FIG. 26.—Ocular Filar Micrometer for Very Exact Measurements. By means of a micrometer screw a line is moved across the field. The distance is measured by means of the divisions on the drum.

aqueous-alcoholic dyes stand next in preference, *never strong stains* like carbol-fuchsin, anilin-water dyes, or saturated alcoholic solutions of dyes.

II. Using a Filar Ocular Micrometer. The filar ocular micrometer is an instrument for the accurate measurement of microscopic objects. It consists of an ocular, between the eye and field lenses of which there is a scale ruled on glass in millimeters and half millimeters, below

and across which a single-line index is made to travel by the use of the micrometer screw.

The micrometer screw is fitted with a drum divided into 100 parts, one revolution of which moves the index line one division or 5 microns. The drum is divided into fifty parts, so that each mark on the drum scale corresponds to $\frac{5 \text{ microns}}{50}$ or 0.1 micron.

The micrometer value of each interval should be calibrated for each objective with the aid of the object micrometer. The eye-lens of the micrometer is adjustable to enable the observer to focus the scale accurately.

The filar ocular micrometer slips into the draw-tube of the microscope like any ordinary ocular and may be fixed in position by the milled-head screw on the side.

A. Calibration of the Filar Ocular Micrometer.

Apparatus. Filar ocular micrometer; microscope; object micrometer.

Method. 1. Place the object micrometer under objective No. 3 and ocular No. 1, drawing out the draw-tube to 17 mm.

2. Bring the lines on the object micrometer into a sharp focus.

3. Replace ocular No. 1 with the filar ocular micrometer.

4. Focus again so that the division of the object micrometer and the ocular micrometer are equally clear and turn the ocular micrometer so that the lines of both micrometers are parallel to each other.

5. Determine how many microns one space of the ocular micrometer represents.

Example. Six divisions of the ocular micrometer-scale cover the same length as three divisions of the object micrometer on which each division is 1/100 millimeter or 10 microns; therefore, six divisions of the ocular micrometer scale equals 30 microns and one division equals 1/6 of 30 or 5 microns.

6. Determine how many revolutions of the drum (from 0 to 0) are necessary to move the movable line one division and from this determination calculate the value in microns, of one division on the drum. One determination of this value is sufficient.

B. Method of Using the Filar Ocular Micrometer.

1. Replace the object micrometer by a slide containing organisms, focus, and measure an organism, counting the number of divisions the drum is turned in moving the movable line from end to end of the organism.

Example. If the drum is turned two divisions the organism was two times 0.1 micron in length or 0.2 micron.

2. To measure the microorganisms with a higher power objective, the value of each division of the scale has to be recalibrated.

EXERCISE 20. DETERMINATION OF THE RATE OF MOVEMENT OF MOTILE ORGANISMS

Apparatus. Microscope; Leitz "step" micrometer; stop-watch; hanging-drop preparation of motile organisms.

Method. Using a hanging-drop preparation of the organism to be examined, determine the rate of movement per second, using the step micrometer and a stop-watch.

EXERCISE 21. PREPARATION OF A HANGING DROP

The purpose of the hanging-drop preparation is to study bacteria in the living condition; to demonstrate (*a*) their form, (*b*) arrangement, (*c*) motility (this is best observed from twenty-four-hour cultures), (*d*) appearance, (*e*) division of cells, (*f*) formation or presence of spores; (*g*) to determine the presence and types of microorganisms in any material and to watch the changes in the predominating types of the microbial flora in a medium from day to day; (*h*) and, in pathogenic bacteriology, to demonstrate agglutination.

Bacteria have two kinds of movement, the so-called Brownian or *molecular movement,* and *true motility.* The former may be demonstrated by examining the movement of powdered carmen rubrum in the hanging drop. A very little of the powder is sufficient. Brownian movement is shown more or less by all small particles of insoluble matter (including living non-motile or dead bacteria) in suspension. It is characterized by a vibratory movement affecting the entire field; the relative positions of the insoluble particles are never altered. This type of move-

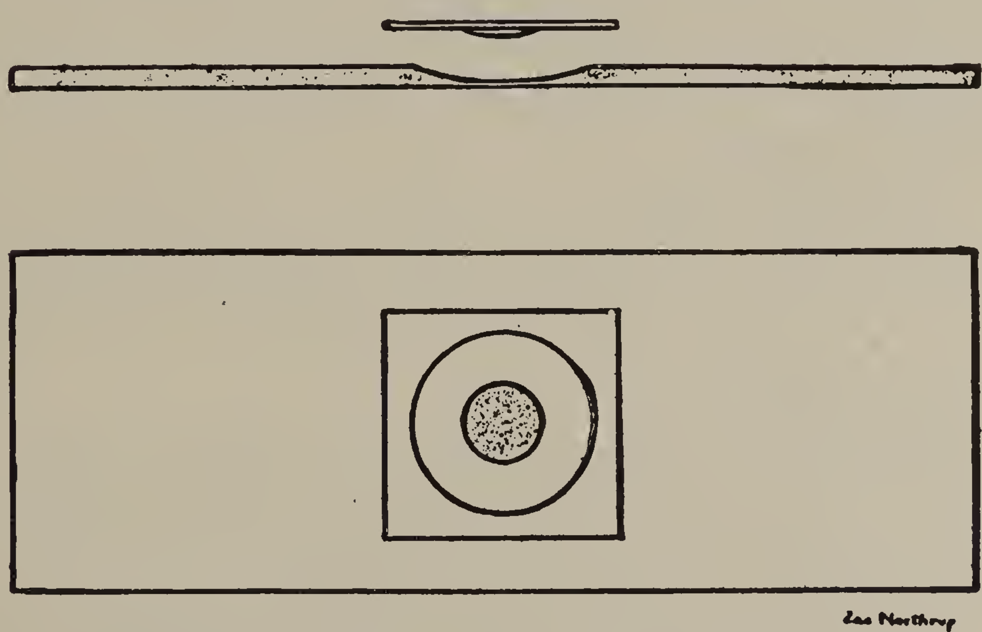


FIG. 27.—Hanging Drop Slide. (Orig.)

ment must be distinguished from that of true motility, which is characterized by the *progressive movement,* more or less rapid, of an organism across the field of the microscope, changing its position in the field independently of and in a direction contrary to other organisms present.

There should be no currents of air entering under the cover-glass and passing through the concavity of the slide nor should there be currents in the liquid. The latter may occur if the organisms have not been well mixed through the drop in the process of preparation. If large numbers of the microorganisms in the drop are moving *in one direc-*

tion, this is an indication of currents in the liquid which have been induced by the liquid touching the side of the concavity, by the drop being too large, by improper mixing, or by air currents; this fault may be remedied by thoroughly mixing the bacteria in the drop with the straight needle or by resealing the cover-glass upon the slide.

Apparatus. Clean cover-glasses; clean concave slides; platinum loop; straight platinum needle; Bunsen burner; distilled water; cover-glass forceps; melted paraffin or vaselin if preparation is to be sealed permanently.

Method. 1. With a platinum loop place four small drops of water about the edge of the depression of the concave slide.

2. For cultures:

In liquid media.

(*a*) With a sterile platinum loop transfer a portion of the culture to the center of a clean cover-glass.

On solid media.

(*a*) With a sterile platinum loop place a small drop of water or physiological salt solution in the center of a clean cover-glass.

(*b*) With a sterile platinum needle transfer a minute portion of the culture to the drop of water so that only the *faintest* cloudiness appears.

3. Quickly invert the cover-glass over the depression in the concave slide and gently depress the margin on the water until the chamber is sealed air tight. The hanging drop must not touch the bottom of the concavity. Note the illustration. If it is desired to keep the hanging drop longer than five to ten minutes, it may be sealed with paraffin as with the adhesion culture, or with vaselin.

The drop must remain over the *center* of the concavity. If the drop touches the side of the concavity, the hanging drop as such is destroyed and it will be necessary to remake the preparation. If pathogenic organisms are used, both

slide and cover-glass must be placed in 1/1000 mercuric chloride or some equally efficient disinfectant for at least one hour before cleaning or reusing.

4. Examine first with objective No. 3, then with objective No. 7 or the 1/12 oil immersion lens, using ocular No. 1 in each case. After a perfect focus is obtained, ocular No. 4 may be used if desired.

Manipulation of Microscope. Using the lowest power objective and ocular, focus the tube of the microscope down by means of the coarse adjustment until the objective nearly touches the cover-glass, *being careful not to touch it.* Then, with the eye at the ocular, focus *up* with the coarse adjustment and move the preparation until the *edge* of the drop comes plainly into view. *This focal point may be passed without noticing it if the light is too intense or too dim.* The edge of the drop is a curved line. The preparation should be so moved that this line cuts the center of the field.

Focus up slightly, swing the No. 7 or 1/12 objective as desired, into place and after the field desired is obtained with the coarse adjustment, focus down until the objective nearly touches the cover-glass. Then with the eye at the ocular, focus up carefully with the coarse adjustment until the edge of the drop comes plainly into view. Use the fine adjustment to bring out details.

In using the 1/12 oil immersion lens a small drop of immersion oil is placed in the center of the cover-glass, the 1/12 objective swung into place as above. *Greater care must be exercised in focusing, as this objective has a shorter working distance.*

EXERCISE 22. PREPARATION OF THE ADHESION CULTURE

The purpose of the exercise is to show the germination of mold spores or the budding of yeast cells, i.e., colony formation.

Apparatus. Clean cover-glasses; clean concave slides; melted paraffin; small glass rod or camel's-hair brush;

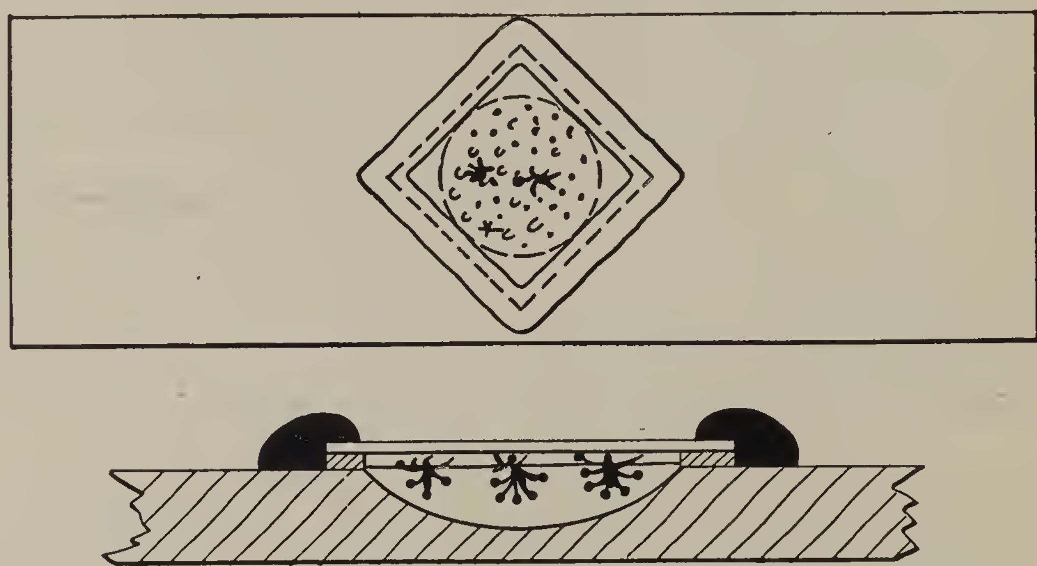


Fig. 28.—Lindner's Adhesion Culture. (Adapted from Lafar's Technische Mykologie.)

sterile cider in tubes. (Wort, milk and other media may be used as conditions demand.)

Cultures. Pure culture of mold, or yeast. If mold spores are to be germinated, an old culture having spores is necessary.

Method. **1.** Inoculate a tube of sterile cider from the pure culture of the organisms to be studied. (Use spores in the case of mold and some of the cells for yeasts.) Distribute well with the platinum needle.

2. Transfer one loopful to a flamed cover-glass and spread in a thin film over the entire surface of the cover-glass, using the straight needle. If any of the cider adheres in droplets, shake them off.

3. Breathe into the concavity of a concave slide until small droplets of moisture are visible on the glass. Before this moisture evaporates and while the cover-glass is still wet turn the cover-glass, culture side down, cornerwise, covering the concavity on the slide.

4. Using the small glass rod or a camel's-hair brush dipped in hot paraffin, neatly seal the cover-glass on the slide so that the cavity will be air tight and the moisture will be retained. Success depends largely on quick work.

5. Examine with objective No. 7 and ocular No. 1. There should be five to twenty spores or cells on a slide. If more are found, a new culture should be made. It may be necessary to inoculate a second tube of cider from the first to secure the proper dilution.

6. If you are not familiar with the spores or cells of the organism to be studied, before making an adhesion culture, mount them in a drop of water heavily inoculated, cover with a cover-glass and examine microscopically.

7. Keep the cultures at room temperature. Examine as often as possible for thirty-six hours and then every twenty-four hours till growth ceases.

8. *Draw as many stages as possible.* The time required for spore germination is usually six to forty-eight hours.

Note. Some molds grow quite extensively in the adhesion culture, even producing fruiting bodies. *Very often both the mycelium and fruiting bodies show peculiar abnormalities and should never be drawn to represent normal structures.* These abnormalities are the result of the peculiar environment.

9. Failure to obtain growth of the mold spores or yeast cells may be due to imperfect sealing, insufficient moisture at the start or *too many cells* on the cover-glass. If the adhesion culture fails to grow, a fresh tube of cider must be inoculated before making new adhesion cultures, as the food materials contained in the medium are partly or entirely used. In the case of mold spores it is reasonable to expect that any mold spores in the adhesion culture

will have germinated within forty-eight hours after preparing the mount.

Note. This method may be utilized to study the colony development of bacteria also.

EXERCISE 23. PREPARATION OF THE MOIST-CHAMBER CULTURE

The purpose of the exercise is to study colony formation in molds, yeasts and bacteria.

Apparatus. Clean cover-glasses; small glass rings,

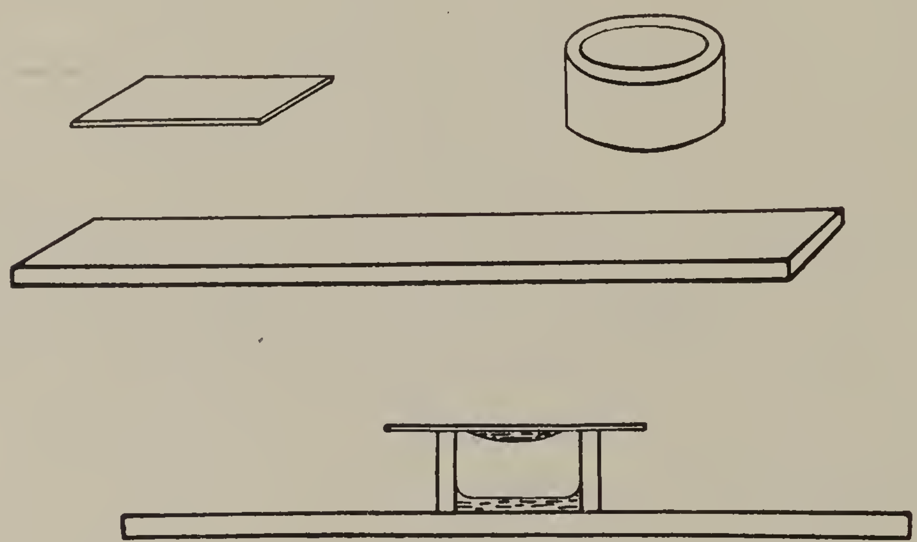


Fig. 29.—Moist Chamber Culture. (Orig.)

clean; clean slides; sterile pipette; paraffin or vaselin; sterile distilled water in test tube; tube of a sterile liquid nutrient medium; platinum needle and loop; forceps; cover-glass.

Culture. Pure culture of organism to be studied.

Method. **1.** With forceps, carefully sterilize in a flame a glass slide and a glass ring designed for this purpose. This should be done by a swinging motion to insure uniform distribution of the heat.

2. Around the edges of the ring, after it has cooled sufficiently, place a little vaselin, while the ring is still held in sterile forceps.

3. Place the ring on the slide and press it down gently to make contact complete. The vaselin renders the chamber water tight.

4. Seal the ring to the slide with melted paraffin as in the adhesion culture, to keep it from slipping around.

5. With a sterile pipette convey into this chamber just enough (boiled) water to cover the bottom.

6. Vaselin the upper edge of the ring.

7. Inoculate lightly the tube of liquid medium with the organism to be studied. Distribute throughout the liquid with the needle.

8. Transfer one loopful to a cover-glass.

9. Using the straight needle, spread in a thin film over the entire surface of the cover-glass. If any of the liquid adheres in droplets, shake them off.

10. Press the cover-glass, medium side down, upon the upper vaselined edge of the ring.

11. Seal the edge of the cover-glass to the glass ring in several places with paraffin to prevent it from slipping around.

12. Incubate at the desired temperature.

This possesses some advantages over the adhesion culture, as more air and moisture and consequently more favorable conditions are furnished for growth. With a little more delicate manipulation agar or gelatin can be used in place of the liquid medium.

EXERCISE 24. PREPARATION OF AGAR HANGING-BLOCK CULTURE

This method was devised by Hill * for studying to better advantage the morphology and manner of multiplication of bacteria.

Carry out this procedure in a special plating room or chamber if possible, to avoid contamination from air currents.

* Hill, Journal of Medical Research, Vol. VII, March, 1902, p. 202.

Apparatus. Clean cover-glasses; clean concave slides; ordinary slides, clean; tube of sterile nutrient agar or gelatin; paraffin; two sterile Petri dishes; scalpel; platinum loop.

Culture. Pure culture of the organism to be studied.

Method. **1.** Liquefy a tube of nutrient agar or gelatin, pour it into a sterile Petri dish to the depth of about 4 mm. and allow it to harden.

2. With the flame-sterilized scalpel, cut out a block of agar about 8 mm. square.

3. Raise the agar block on the blade of the scalpel and transfer it, under side down, to the center of a sterile slide.

4. With a sterile platinum loop, spread a drop of the liquid culture (or suspension of organisms from a solid culture medium) over the upper surface of the agar block as if making a cover-glass film.

5. Place the slide and block in a sterile Petri dish and incubate for ten minutes at 37° C. to dry slightly.

6. With sterile forceps, lower a clean, dry, sterile cover-glass carefully on the inoculated surface of the agar (avoiding air bubbles), so as to leave a clear margin of cover-glass overlapping the agar block.

7. Invert the preparation and, with the blade of the scalpel, remove the slide from the agar block.

8. With the platinum loop, run a drop or two of melted agar around the edges of the block. This solidifies at once and seals the block to the cover-glass.

9. Sterilize a concave slide.

10. Invert the cover-glass with the block attached on the concave slide and seal it in place, firmly, with paraffin.

11. Observe immediately and later from time to time with ocular No. 1 and objective No. 7 or the oil immersion lens.

EXERCISE 25. LINDNER'S CONCAVE-SLIDE METHOD FOR DEMONSTRATING FERMENTATION

The object of this exercise is to test the fermenting power of yeasts.

Apparatus. Three clean concave slides; three clean cover-glasses; sterile filter paper (place several pieces 8 cm. square in a Petri dish and sterilize in the hot air); three tubes of sterile wort or cider; three sterile 1 c.c. pipettes; forceps; melted paraffin; platinum needles; Bunsen burner.

Cultures. *Saccharomyces cerevisiæ; Saccharomyces apiculatus; Torula rosea.*

Method. The following procedure is to be used for each organism to be tested:

1. Using the straight needle, inoculate a tube of wort with *Saccharomyces cerevisiæ* and mix well through the medium.

2. Sterilize a concave slide in the flame.

3. Using a sterile pipette, fill the concavity of the slide until the liquid " rounds up " over the concavity.

4. Holding a cover-glass in the forceps, sterilize it in the flame.

5. Lay the cover-glass on the end of the slide and push it over with the forceps until the cover-glass covers the concavity, thus sealing in the inoculated liquid. *There must be no air bubbles. The preparation must be made over again if this occurs.*

6. Remove the excess liquid with sterile filter paper, using forceps to hold the paper.

7. Seal the cover-glass with paraffin as with the adhesion culture.

8. Place the slides in a horizontal position in Petri dishes, or in a slide box as convenient.

9. Incubate at 25° to 30° for twenty-four hours. Gas bubbles will be formed in twenty-four to forty-eight hours, if any fermentation occurs.

10. Record the time of fermentation and the relative fermentation of each yeast and draw conclusions from your results.

11. Do your results coincide with those in the references given?

12. State in detail your results with any conclusions which follow from them, and point out the practical applications which may be made.

By the use of sugar broth in place of wort, this method may be employed for bacteria as well.

REFERENCES

LAFAR: Technical Mycology, Vol. II, Part 1, pp. 113, 114, and Part 2, pp. 401–407, 430–436. (Index of three volumes is in Vol. II, Part 2.)

GREEN: Soluble Ferments and Fermentation, pp. 333–362.

CONN: Bacteria, Yeasts and Molds, pp. 56–99.

EXERCISE 26. LINDNER'S DROPLET CULTURE

The object of the exercise is to isolate a single yeast cell and watch its development.

Apparatus. Sterile cover-glass (sterilize in flame); con-

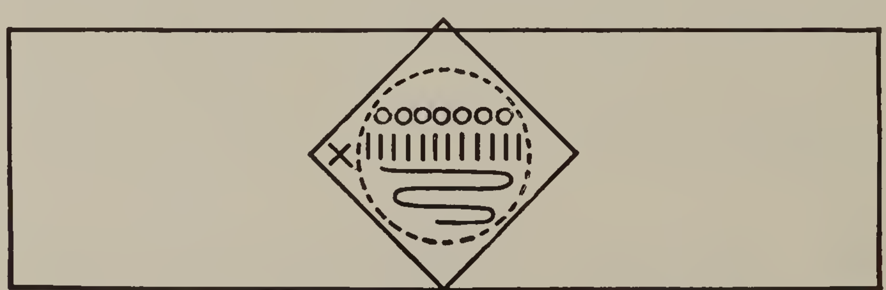


FIG. 30.—Lindner s Droplet Culture. (Adapted from Lafar's Technische Mykologie.)

cave slide; forceps; sterile toothpick (sterilize in a test tube in hot air); paraffin; India ink.

Culture. Pure culture of some yeast.

Method. **1.** Inoculate a tube of cider with yeast. Distribute the organisms well.

2. Using the sterile toothpick, make five rows of small droplets (five droplets in a row) on a sterile cover-glass and place, culture side down, over the concavity of a sterile slide.

3. Seal the cover-glass with paraffin as in the preparation of the adhesion culture. Examine microscopically.

4. Locate one droplet which contains only one cell. Using India ink, write the location of this droplet on the slide.

5. Make a drawing of each stage of development until growth ceases. Why does the cell stop growing?

6. State in detail your results with any conclusions to be drawn and point out the practical applications which may be made.

This method may be used to advantage with mold spores.

EXERCISE 27. CHINESE INK PREPARATION

Chinese ink may be used to make bacteria more easily visible microscopically and to aid in taking correct measurements.

Apparatus. Sterile, dilute Chinese ink; * clean flamed glass slides.

Cultures. Pure cultures (young agar streaks are best).

Method. 1. Place one loopful of distilled water and three loopfuls of sterile Chinese ink in a row on a clean glass slide, about 2 cm. apart.

2. Inoculate the loopful of water from the original culture.

3. Distribute the organisms well with a platinum needle.

4. Then inoculate the adjoining drop of ink from the loopful of water, the second drop of ink from the first, etc.

5. Stir each loopful of ink well and then spread it so as to cover an area about 1 cm. square.

* See appendix for method of preparation.

6. Let dry. If desired the specimen may be mounted in Canada balsam before examining.

7. Examine with either the 1/7 or the oil immersion objective.

8. Write the name of the organism, the date, and your name on the glass with India ink.

By the use of the Chinese ink preparation, it is possible to examine any organism unstained. Organisms so treated neither shrink nor in any way change their form, making

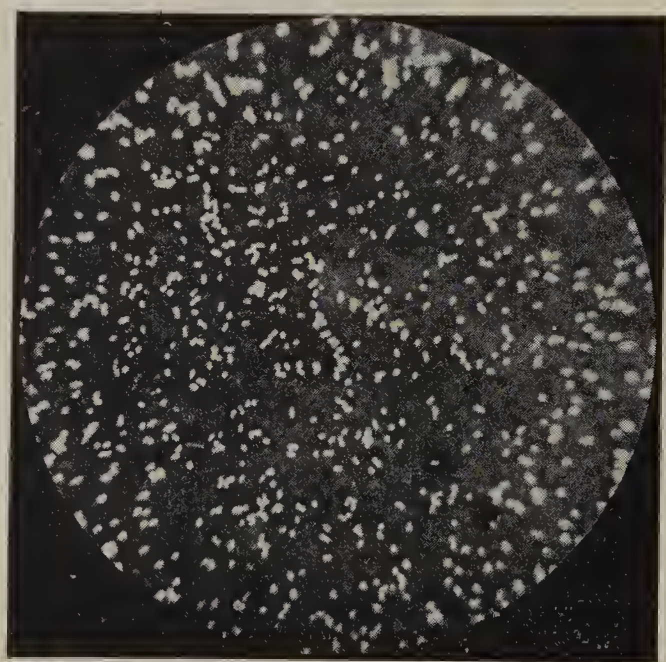


Fig. 31.—Chinese Ink Preparation. (Orig. Northrup.)

accurate measurement possible. Stains often cause organisms to appear swollen or shrunken.

The motility of bacteria may be more easily demonstrated by adding a very slight amount of this ink to a hanging drop of the organism being studied.

Caution. *Chinese ink is very expensive.* When making preparations, use every precaution to keep your supply sterile, as contaminating organisms may be confused with the culture under study. A control preparation to which no microorganisms have been added will serve to detect their presence.

EXERCISE 28. THE STAINING OF MICROORGANISMS

Microorganisms are devoid of color as a rule and are stained for the purpose of observing their morphology to better advantage than in a hanging drop. Staining also often serves to bring out certain morphological characteristics which are otherwise not evident, such as the presence of metachromatic granules or a peculiar arrangement of the protoplasm, resulting in what are known as "beaded forms."

The stains best suited to bacteria are the basic anilin dyes which are derived from the coal-tar product anilin ($C_6H_5NH_2$). Many of them have the constitution of salts.

Such compounds are divided into two groups, according as the staining action depends on the basic or the acid portion of the molecule. Fuchsin, gentian violet and methylen blue are basic dyes, while eosin, picric acid and acid fuchsin are acid dyes.

These groups have affinities for different parts of the living cells. The basic stains have a special affinity for the nuclei of tissues and for bacteria, the acid for the protoplasm and not for bacteria. The violet and the red anilin dyes in order, are the most intense in action, easily overstaining the specimen. It is difficult to overstain with methylen blue. For this reason this stain is to be preferred where the bacteria occur in thick or viscid substances, like pus, mucus or milk. In the presence of alkali, however, the stain acts more energetically.

Stock solutions of the ordinary dyes are commonly used. These are prepared by making a saturated solution of the dye in absolute alcohol; this is diluted with water as needed.

Saturated alcoholic solutions of dyes will stain bacteria with difficulty. The best results are obtained with the diluted stain, spoken of here as an "aqueous-alcoholic" stain.

Apparatus. Clean cover-glasses; clean slides; cover-glass forceps; platinum loop and needle; Bunsen burner; small pieces of filter paper; distilled water; aqueous-alcoholic solution of fuchsin, methylen blue, etc.; Canada balsam; microscope.

Note. See appendix for formulæ of stains.

Method. **1.** Flame a clean cover-glass, holding it by one corner with cover-glass forceps.

2. Place one loopful of distilled water in its center.

3. Touch the growth on slant agar *lightly* with a sterilized platinum needle and transfer a *very little* of the material to the drop, and only sufficient to make it very slightly cloudy.

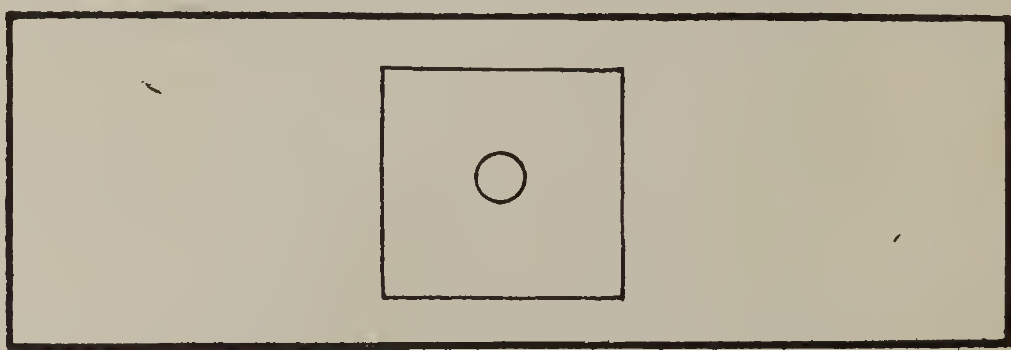


Fig. 32.—For use in mounting permanent cover-glass preparations. (Orig.)

4. Flame the needle and allow it to cool.

5. Spread the drop over the entire cover-glass with one or two strokes of a straight needle. In the case of *pathogenic* microorganisms use a flat loop 2 mm. in diameter, and limit the spreading to the inner three-fourths of the cover-glass.

6. Allow to *dry* in air.

7. Fix the preparation on the cover-glass by passing the cover-glass, specimen side up, three times through the flame of a Bunsen burner. The speed is measured by moving the cover-glass and forceps in a circle of 1 ft. diameter in one second.

8. Flood the entire specimen-side of the cover-glass with stain, using a pipette.

9. Allow the stain to act a short time.

Note. The time required for staining varies so much with the different stains, different organisms and their physiological conditions, that no exact time can be given. In general, a good specimen is obtained by staining one-half to one minute with fuchsin or gentian violet, or one to five minutes with methylen blue.

10. Wash the specimen in running water.

11. Mount the cover-glass in water, specimen side down, on a clean slide.

12. Dry the upper surface of the cover-glass and take up any excess of water by means of filter paper.

13. Examine the slide under the microscope, using objective No. 7 and ocular No. 1.

14. If satisfactory, remove the cover-glass carefully from the slide, floating it off if necessary.

15. Allow it to dry in the air, specimen side up.

16. Place a clean slide exactly on the figure (Fig. 32).

17. Let a small drop of Canada balsam fall in the center of the slide, marked by the circle.

Note. The consistency of the Canada balsam should be like thin cream. The diameter of the glass rod should not be more than 4 mm.

18. Place the cover-glass, specimen side down, on this drop.

19. Allow the balsam to spread over the entire under surface of the cover-glass (without pressing it down on the slide) and keep the cover-glass straight, coinciding with the lines of the figure.

20. Label, stating in order, the name of the organism, the age and kind of culture, the stain used, the date, your own name and the purpose of the stain if otherwise than ordinary, e.g., spore stain.

21. Allow the slide to stand in a horizontal position for a few days until the balsam becomes hard.

EXERCISE 29. ANJESZKY'S METHOD OF STAINING SPORES

Spores are not stained by the ordinary staining methods, as their physical nature differs from that of the vegetative rods within which they are formed. By proper treatment with strong anilin dyes, however, it is possible to force the stain into the spore. Once within the spore it is as difficult to remove the dye as it was to cause it to enter.

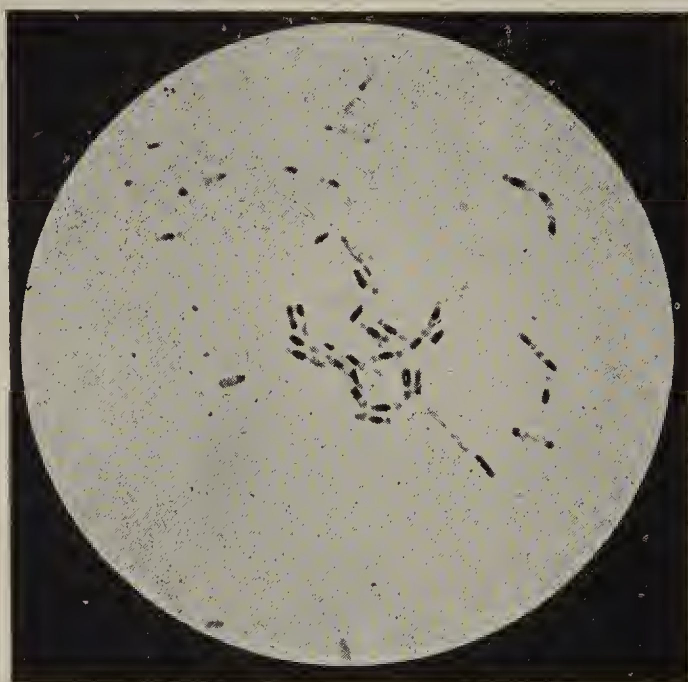


Fig. 33.—Contrast Spore Stain, Carbol-fuchsin and Methylen Blue, ×1500. (Orig. Northrup.)

By a careful decolorization with a weak acid, it is possible to remove the stain from everything on the cover-glass except from the spores. Then, on application of a dye of a contrasting color, the specimen will show, e.g., a bright red spore within a blue bacterium.

The fundamental principles of this method are also used for staining "acid-fast" organisms, as *Bact. tuberculosis*.

Apparatus. Clean cover-glasses; clean slides; cover-glass forceps; platinum loop and needle; Bunsen burner;

carbol-fuchsin; methylen blue, aqueous-alcoholic; hydrochloric acid, 0.5%; sulphuric acid, 5%; Canada balsam; microscope.

Culture. Culture of an organism just beginning to show spore formation.

Method. **1.** Prepare a cover-glass film of the spore-containing organism and allow it to dry.

2. While it is drying, warm some 0.5% HCl in the small evaporating dish over a Bunsen burner until it steams well and bubbles begin to form.

3. When the solution is hot and the smear dry, drop the cover-glass upon the fluid and allow it to act upon the unfixed smear for three to four minutes.

4. Wash and dry the cover-glass.

5. Fix in the flame *for the first time.*

6. Stain with carbol-fuchsin by flooding the cover-glass with the stain, warming twice until fumes arise.

7. Allow to cool, and wash in water.

8. Decolorize with 5% H_2SO_4. Spores are treated with a mild decolorizing agent, as they are much less resistant to acid than are acid-fast bacteria. (See p. 91, step 7.)

9. Wash in water.

10. Counterstain for one to two minutes with methylen blue.

11. Wash, dry and examine the specimen in water. If satisfactory, dry it and mount in balsam.

The whole procedure should not take longer than eight to ten minutes.

REFERENCE

McFarland: Textbook of Pathogenic Bacteriology, p. 188.

EXERCISE 30. METHOD OF STAINING TUBERCLE AND OTHER ACID-FAST BACTERIA

Acid-fast bacteria are so termed from their reaction to a special staining process. This process consists in staining the specimen containing, for example, tubercle bacteria, with hot carbol-fuchsin and decolorizing for a short time with acid; the acid takes the dye out of all other material, bacteria and blood or other body cells that may be present, leaving the tubercle bacteria stained red. This staining process is essentially the same as for spores, but the principle is different.

The property which some bacteria possess of being acid-fast is attributed to the presence of fat and wax-like substances in their cells. This seems to be proved by the fact that when the bacterial cell substance of tubercle bacteria has been freed from these fats and waxes by extraction with absolute alcohol and ether, this property is lost.

Apparatus. Clean slides; clean cover-glasses; platinum loop; copper staining dish; Bunsen burner; forceps; carbol-fuchsin; sulphuric acid, 20%; methylen blue, aqueous-alcoholic; immersion oil; Canada balsam; specimen to be examined.

Method. 1. Using a sterile loop, smear some of the specimen in the center of one surface of a clean slide, taking care not to come within 0.5 cm. of the edge.

Note. This may be applied to sputum, pus, etc. In case of tubercles or diseased organs or tissues these may be cut open with a scalpel, a portion incised, and grasping this portion with the forceps a smear made directly on the slide, following the precautions above. If pure cultures are to be examined, a cover-glass specimen may be made in the usual way.

2. Dry the slide in air.

3. Fix in the flame.

4. Support the slide on the copper staining dish; flood

the slide with carbol-fuchsin until the stain "rounds up."

5. Heat the under side of the slide directly with a flame until the carbol-fuchsin steams (*but not boils*). Keep the stain steaming for five minutes.

6. Wash in water.

7. Decolorize by dipping the slide preparation into 20% H_2SC_4 *for an instant* and washing *immediately*. This process may have to be repeated two or three times. If not careful, however, the tubercle bacteria may be decolorized. If this happens, their acid-fast property will be destroyed to some extent.

8. Counterstain with aqueous-alcoholic methylen blue.

9. Wash in water, dry and examine directly with the oil immersion lens. The specimen, if a good one, may then be mounted in the usual way without removing the immersion oil.

EXERCISE 31. METHOD FOR STAINING FLAGELLA

Flagella, the exceedingly delicate organs of locomotion of bacteria, cannot be seen in an unstained or in an ordinary stained preparation. Special staining methods must be employed to make them visible. They are generally rendered visible by precipitating some chemical on them; this generally increases their width considerably.

The staining of the flagella of bacteria is the most difficult of all bacteriological procedures and it generally requires considerable practice to insure good results.

There are many methods for staining flagella. This one, however, has met with considerable success with students. Failure to make a good flagella stain with any method is no sign that the student is not a good workman, nor is success the sign of a good bacteriologist.

Apparatus. Clean glass slides; *absolutely clean* cover-glasses; small platinum loop; several cover-glass forceps;

distilled water; mordant for flagella staining; anilin-water fuchsin or gentian violet.

Culture. Agar slant culture, twelve to eighteen hours old.

Note. The best results are obtained if successive generations of this organism have been transplanted every eighteen to twenty-four hours for several generations.

Method. **1.** Place three drops of distilled water on a clean glass slide.

2. Transfer a small amount of bacterial material from

FIG. 34.—Flagella Stain. *B. typhosus*, ×1500.

the moist portion of the agar slant culture to the first drop by means of a small platinum loop, using only enough of the material to make this drop very slightly cloudy.

3. Flame the needle and transfer a small portion from the first drop to the second.

4. Proceed in like manner in preparing the third dilution.

5. Place a number of *absolutely clean* cover-glasses in cover-glass forceps.

6. By means of a platinum needle bent at right angles near the end, make smears on the cover-glasses from the

second and third drops on the slide by drawing the bent needle *once*, *lightly* across the cover-glass. *If there is any tendency of the smear to roll or "gather in drops," the cover-glass should be discarded and a clean one substituted.* This is imperative.

7. Allow the preparation to dry for about five minutes.

8. Filter on each of the fixed smears enough of the mordant to cover the cover-glass.

9. Allow to stand about five minutes at room temperature.

10. Wash off the mordant in a small stream of water.

11. Draw off the excess water from the edge of the cover-glass by means of filter paper.

12. Stain with anilin-water fuchsin or anilin-water gentian violet for about five minutes, either cold or by warming somewhat over a low flame.

13. Wash off the excess stain with clean water.

14. Mount on a slide in water.

15. Absorb the excess water with filter paper.

16. Examine under the microscope. If the preparation has been successful, it may be dried and mounted in balsam.

EXERCISE 32. GRAM'S METHOD OF STAINING

Certain organisms, when stained with anilin-water gentian violet and afterwards treated with a solution of iodin and washed in alcohol or anilin oil, give up the stain; others retain the color when subjected to this process. These latter organisms are said to be *Gram-positive*, those losing the stain are *Gram-negative*.

This phenomenon is interpreted by Benians to be due to the possession of a definite cell-envelope which by the action of iodin is rendered impermeable to alcohol. His

experiments show that so long as the Gram-stained cell is intact, the solvent is unable to remove the stain, but that as soon as the cell is crushed and injured, the stain is, in great part, dissolved out. The amorphous débris obtained from broken-up Gram-positive bacteria does not retain Gram's stain.

Apparatus. Clean slides; clean cover-glasses; platinum loop and needle; cover-glass forceps; distilled water; anilin-water gentian violet; Lugol's iodin solution; aceton-alcohol.

Culture. Agar slant cultures preferably.

Method. **1.** Prepare a cover-slip film and fix in the usual way.

2. Stain in anilin-water gentian violet or stabilized gentian violet three to five minutes.

3. Wash in water.

4. Treat with Lugol's iodin solution until the film is black or dark brown.

5. Wash in water.

6. Dry in air.

7. Wash in aceton-alcohol until no more color is discharged.

8. Wash in water. (Counterstain at this point if desired.)

9. Dry in air.

10. Mount in Canada balsam.

Note. The Gram-Weigert method is more applicable in case of sections of tissues. The directions from 1–6 are the same. The specimen is washed in anilin oil 1 part, xylol 2 parts, instead of alcohol, washed further in xylol and mounted at once in Canada balsam. *Bact. bulgaricum* in milk is very beautifully demonstrated by this modified method.

A few of the more common Gram-positive and -negative organisms are appended. This is not as important a diagnostic method as has been formerly supposed, because the reaction occurring often depends upon the age of the culture, the medium on which it is grown, etc.

Gram-positive Organisms.	Gram-negative Organisms.
Staph. pyogenes aureus and *albus*	*Bact. mallei*
Strept. pyogenes	*Bact. aerogenes*
Bact. anthracis	*B. typhosus*
Bact. tuberculosis	*B. coli communis*
B. alvei	*B. choleræ suis*
B. tetani	*M. gonorrheæ*
Bact. acidi lactici	*Msp. deneke*
Bact. bulgaricum	*Msp. finkler-prior*
B. megaterium	*Spirocheta obermeieri*
B. subtilis	*Proteus vulgaris*
B. mycoides	*Ps. medicaginis*
B. mesentericus vulgatus	*B. amylovorus*
M. tetragenus	*Ps. campestris*
Streptothrix actinomyces	*B. phytophthorus*
Sacch. cerevisiæ and other yeasts	*B. caratovorus*
Molds	

REFERENCE

Benians, T. H. C.: "Observations on the Gram-positive and Acid-fast Properties of Bacteria." Jour. of Path. and Bact., Vol. XVII, pp. 199–211 (1912).

EXERCISE 33. METHOD FOR STAINING CAPSULES

Some bacteria possess a gelatinous envelope or "capsule" which in some species surrounds each individual organism, and in others, groups of organisms. The presence of this capsule may be demonstrated by various special staining methods. The capsule takes the stain much less quickly than does the organism, leaving a light-colored halo about it. The presence of a capsule does not always indicate that the organism forming it is a slime-forming organism, nor does the fact that an organism is a slime-former preclude the possession of a capsule.

Apparatus. Clean cover-glasses; clean slides; platinum loop; cover-glass forceps; filter paper, pieces; glacial acetic acid; gentian violet, aqueous-alcoholic.

Cultures. Cultures in milk, serum, etc., media.

Method. 1. Prepare the cover-glass specimen directly from the medium *without the use of water.* Spread and fix in the usual manner.

2. Flood the specimen side of cover-glass with *glacial* acetic acid.

3. Drain immediately without washing. A piece of filter paper may be touched to the edge of glass to take up surplus water and facilitate drainage.

4. Stain with aqueous-alcoholic gentian violet for a few seconds.

5. Examine under the microscope.

6. Wash, dry and mount.

EXERCISE 34. METHOD OF MAKING IMPRESSION PREPARATIONS

Impression preparations (Klatschpräparat) are prepared from isolated colonies of bacteria in order that their characteristic formation may be examined by higher powers than can be used with the living cultivation *in situ.* They are prepared from plate cultivations. Young colonies of *Bact. anthracis* produce beautiful impression preparations.

Apparatus. Clean cover-glasses; clean slides; Novy cover-glass forceps; dissecting needle; stain.

Culture. Agar plate culture containing well-isolated colonies of organism to be studied.

Method. 1. Taking a clean cover-glass in the Novy forceps, open the plate and rest one edge of the cover-glass on the surface of the medium a little to one side of the selected colony.

2. Lower it carefully over the colony until horizontal. *Avoid any lateral movement or the inclusion of air bubbles.*

3. Press gently on the center of the upper surface of the cover-glass with the points of the forceps to insure perfect contact with the colony.

4. Steady one edge of the cover-glass with the forceps and pass the point of the dissecting needle just under the opposite edge and raise carefully; the colony will be adherent to it.

When nearly vertical, grasp the cover-glass with the forceps and remove it from the plate. Re-cover the plate.

5. Place the cover-glass specimen side up on desk and cover with half a Petri dish until dry.

6. Fix in the flame.

7. Stain and mount as with ordinary cover-glass specimen, being careful to perform all washing operations with extreme gentleness.

EXERCISE 35. METHOD OF STAINING THE NUCLEI OF YEAST CELLS

The nuclei of yeast cells are not visible in unstained or in ordinary stained specimens. A special method of procedure must be used.

Apparatus. Clean cover-glasses; clean slides; forceps; ferric ammonium sulphate, 3% aqueous solution; Ehrlich's hematoxylin solution; two staining dishes for slides.

Culture. Culture of *Saccharomyces* or *Torula*.

Method. 1. Prepare and fix the film upon the slide in the usual way.

2. Soak in 3% ferric ammonium sulphate for two hours.

3. Wash thoroughly in water.

4. Stain in hematoxylin solution for thirty minutes.

5. Wash in water.

6. Differentiate in ferric ammonium sulphate solution for one and a half to two minutes, examining wet under the microscope during the process.

7. Wash, dry and mount.

GENERAL CHARACTERISTICS OF MOLD GROWTH AND HINTS FOR STUDY

A brief description of the molds to be studied in the laboratory is here given. The references cited will give more in detail of their structure, importance and occurrence.

In these descriptions, there have been noted the parts of the structure of each mold that are to be found microscopically and drawn, also the quickest method of obtaining the best results. All microscopic drawings and measurements can be secured from the adhesion culture or the moist-chamber culture.

Rhizopus nigricans—Black mold
(*Mucor stolonifer*)

The mycelium in the advanced stage consists of rhizoids (rootlets), bearing clusters of sporangiophores, joined by long hyphæ (the stolons) to the mycelium proper. The hyphæ are non-septate.

The fruiting bodies consist of typical sporangia (spore cases containing spores) borne on the enlarged end (columella) of the sporangiophore. Spores are liberated by the bursting of the sporangium.

The columella can be observed in fruiting bodies of a light brown color; white sporangia are too young and black too old to show this structure. If no fruiting bodies grow in the adhesion culture, they may be studied directly from a plate culture by preparing a glycerin slide. Take care not to burst the sporangium when transferring it to the slide.

Aspergillus niger—Black mold

The mycelium of this mold consists of septate hyphæ with frequent dichotomous branching.

The fruiting body (asexual) consists of an erect condiophore usually ending more or less abruptly in a dilation or head which bears closely packed sterigmata each of which in turn bears a single chain of conidia, the newly formed conidium being pushed away by the formation of a new spore; thus the conidium at the end of the chain is the oldest. The conidia of this mold are black.

Penicillium italicum—Blue-green mold

The mycelium consists of septate hyphæ, having frequent dichotomous branching.

The conidial fructification resembles a brush, the conidia (spores) being borne on the end of conidiiferous cells (sterigmata); in this genus before the conidia appear, there is generally a primary and even a secondary branching of the condiophore in some species before the conidiiferous cells are formed. The species of *Penicillium* have more of a brush-like appearance than the species of *Aspergillus*. The spores of *P. italicum* are blue-green.

Oospora (*Oidium*) *lactis*—White mold

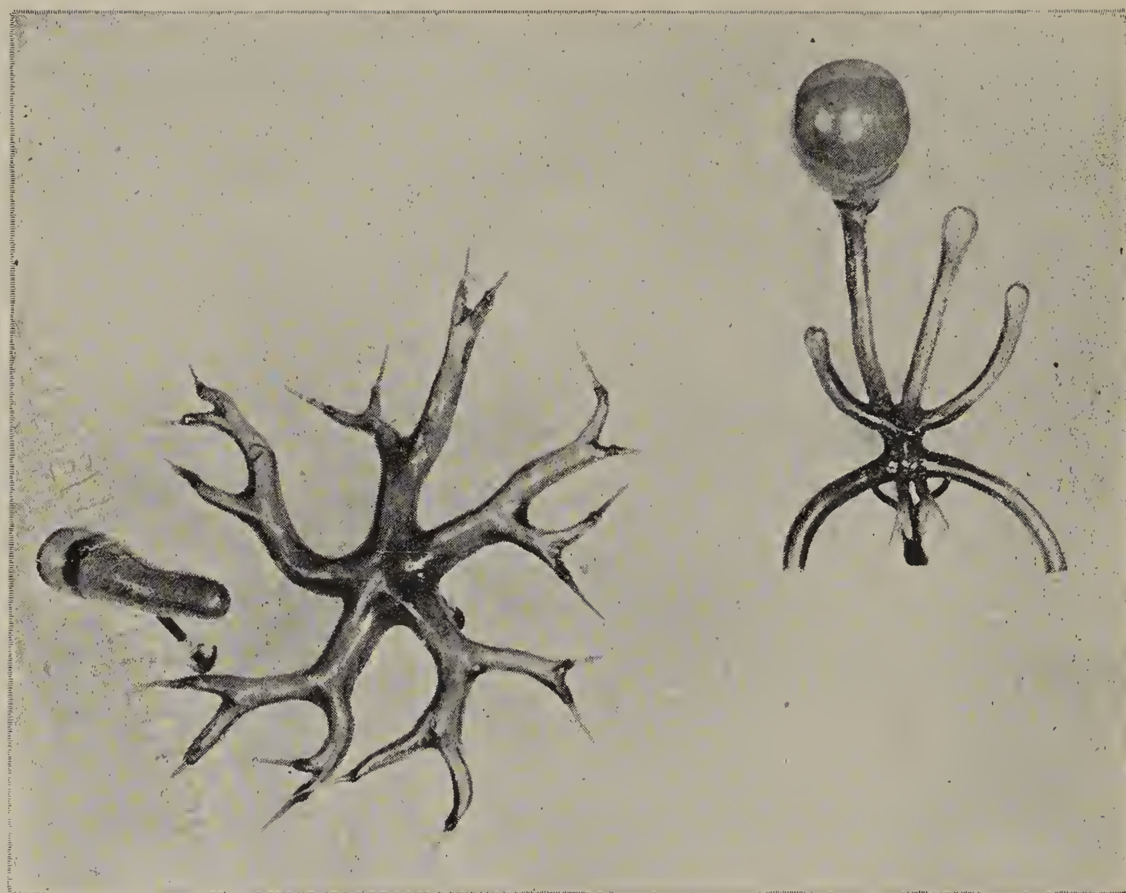
The mycelium consists of septate hyphæ, having dichotomous branching; the hyphæ are almost entirely submerged in the nutrient substrate.

This differs from the other molds in that it does not have typical fruiting bodies. It reproduces by means of conidia, which are formed by a simple division of the hyphæ. The conidia are colorless.

REFERENCES

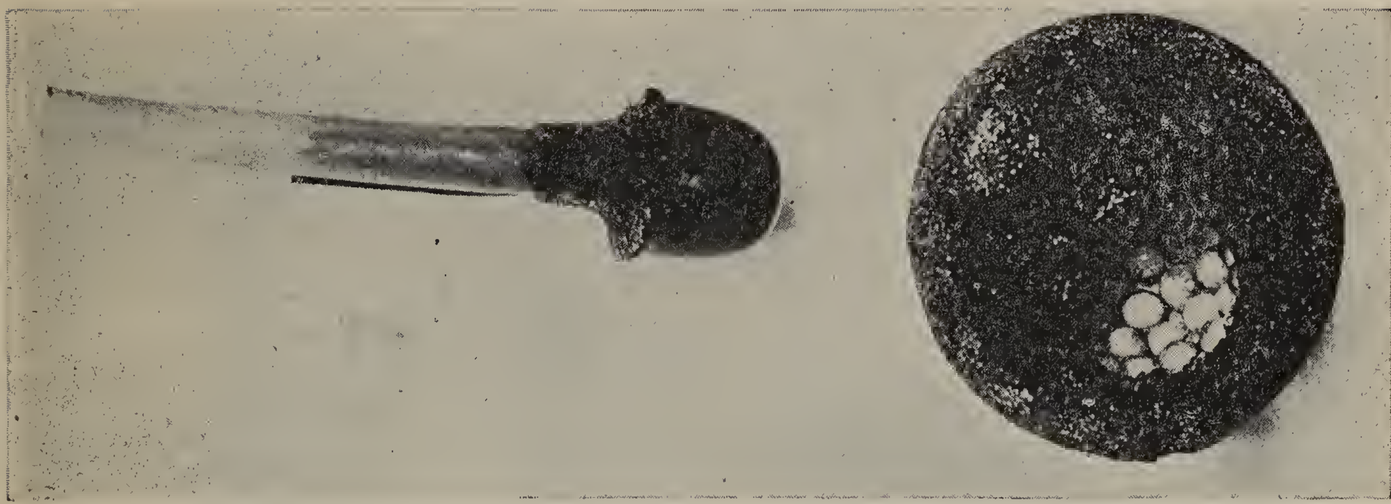
Marshall: Microbiology. Second Edition, pp. 36–58.
Lafar: Technical Mycology, Vol. II, Part I, pp. 5, 15, 71–77; Part II, pp. 300–346, 451–455.
Klöcker: Fermentation Studies, pp. 184, 185, 274–282, 303, 304.

PLATE I*



Germination of *Rhizopus* Spore, Mycelium, Rhizoids and Development of Sporangiophores and Sporangium.

Ripe Sporangium.

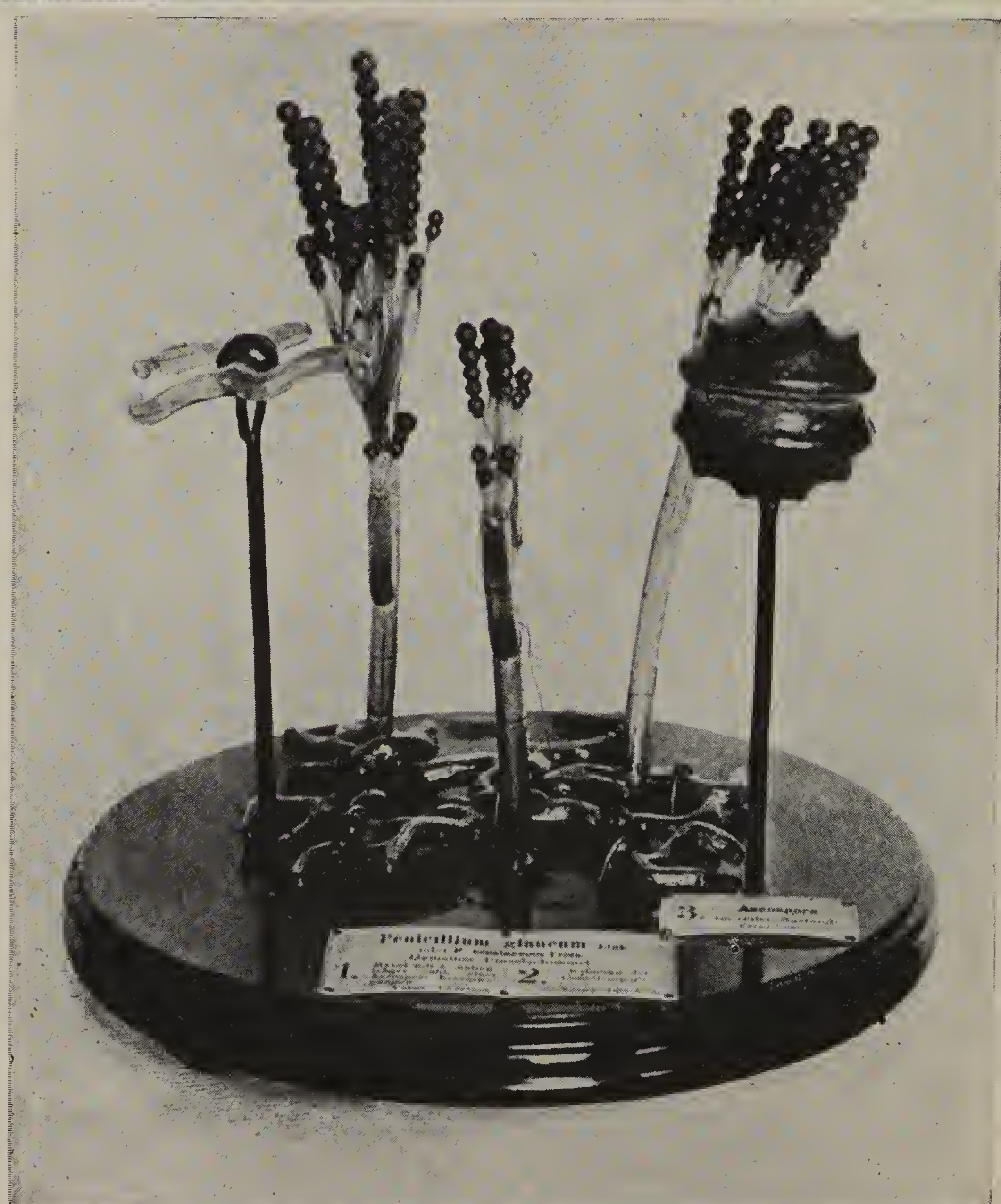


Sporangiophore with Columella Attached and Ripe Sporangium Showing Spores Within.

Various Stages in the Formation and Germination of a Zygospore.

* Plates I–VI photographed from models manufactured by R. Brendel, Berlin—Grunewald, Bismarck—Allee 37, Germany.


Eurotium Aspergillas glaucus de Bary


Penicillium glaucum
1.
2.
3.

EXERCISE 36. MICROSCOPICAL EXAMINATION OF MOLDS

Apparatus. Clean cover-glasses; clean slides; hand lens or compound microscope; platinum needle and loop; dissecting needle; glycerin, 10%.

Cultures. Plate culture of mold.

Method. The gross structure of a mold colony upon a plate may be examined with a hand lens or by placing the inverted Petri dish culture on the stage of the compound microscope and examining with objective No. 3 and ocular No. 1. The structure may be examined in detail as follows:

1. Select a young colony which shows colored fruiting bodies, if such are produced by the organism to be studied. (Growth from natural or artificial media may be treated in the same general way.)

2. Using a sterile platinum needle, transfer a small portion of the mycelium and fruiting bodies to a drop of glycerin on a plain glass slide. If the mold growth is closely confined to the surface of the media (as with *Penicillium* or *Aspergillus*), it is often desirable to cut out a small piece of the medium bearing the mold and lift to the slide by means of a sterile platinum loop.

3. Tease out very gently, using dissecting needles or common pins. The mold structure is extremely delicate, so this operation must be performed with the utmost care.

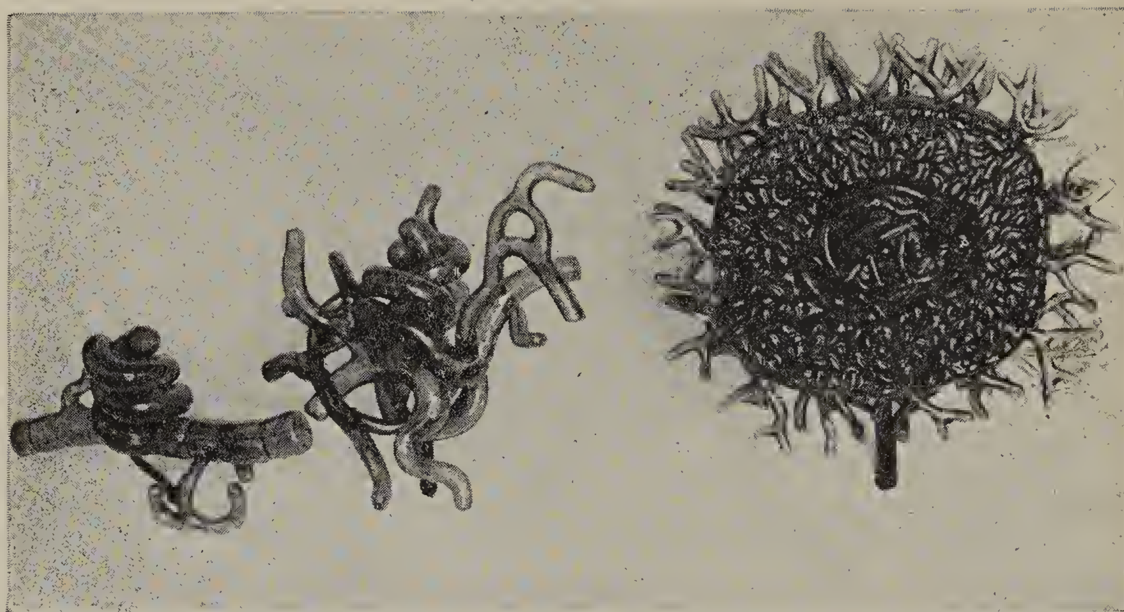
4. Place a cover-glass over the preparation.

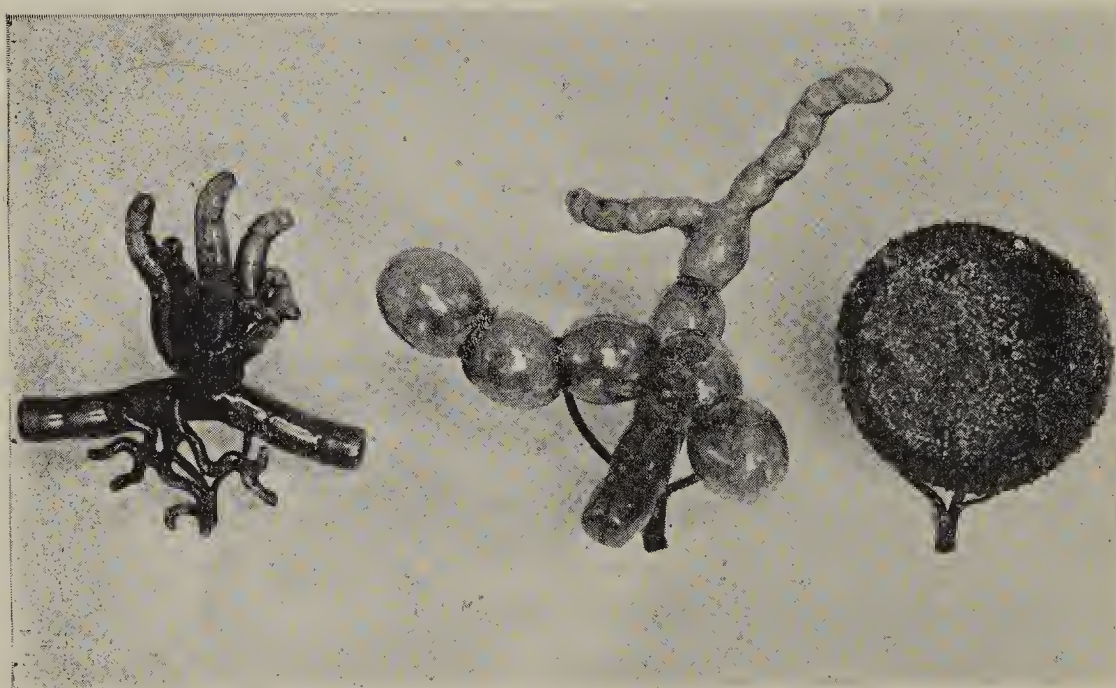
5. Examine with the microscope, using objective No. 3 and ocular No. 1. When a portion of mycelium bearing fruiting bodies is found, examine with objective No. 7. Draw the young and the old fruiting organs.

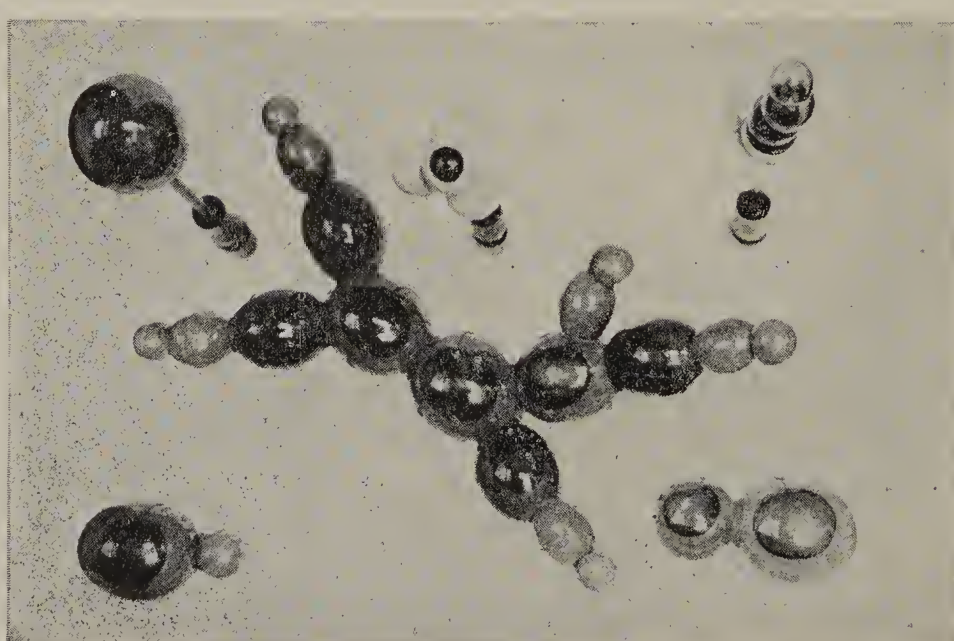
Description of Plate III

Aspergillus, Showing Septate Mycelium, Conidiophore with Conidia, also Formation of Ascogonium.

Penicillium, Germination of Spore, Formation of Mycelium, Septate Conidiophores with Conidia, Ripe Ascospore.







EXERCISE 37. THE STUDY OF MOLDS

Apparatus. Ten sterile Petri dishes; four tubes of sterile slanted agar; ten tubes of sterile agar, for plates; four tubes of sterile cider or wort; four tubes of sterile gelatin; clean glass rings, slides and cover-glasses; hand lens; compound microscope; centimeter scale.

Cultures. Pure or mixed cultures of the following four molds: *Rhizopus nigricans; Aspergillus niger; Penicillium italicum; Oospora lactis.* Mixed (or impure) cultures of two molds growing in their natural habitat will be found on each table.

Method. 1. Plate out each mixed culture* making three straight needle dilution plates for each. Use agar as a medium. Place the plates in the constant-temperature room in the place assigned. Note the temperature.

2. When the plates are twenty-four hours old, mark and draw a well-isolated typical colony of the mold from the most thinly populated plate. Measure and record the diameter of the colony in millimeters.

3. When fruiting bodies begin to show, isolate a pure culture of each mold in cider (see Exercise 16).

4. (*a*) *As soon as growth begins to show* in the tubes of cider (about twenty-four to thirty-six hours) make a macroscopic drawing of each. State the age of the culture.

(*b*) When mycelium is well developed and fruiting bodies appear (as noted on plates) make a second drawing.

*Two mixed and two pure cultures are furnished for study. These cultures owe their color to the presence of fruiting bodies or spores. Always endeavor to obtain spores when making inoculations from molds.

DESCRIPTION OF PLATE IV

Sexual Reproduction of *Penicillium,* Ascus Formation.

Formation of Chains of Asci in Process of Ripening, Some Containing Ascospores, Section of a Ripe Ascus.

Saccharomyces, Budding, Colony Formation, Production of Ascus and Germination of Ascospores.

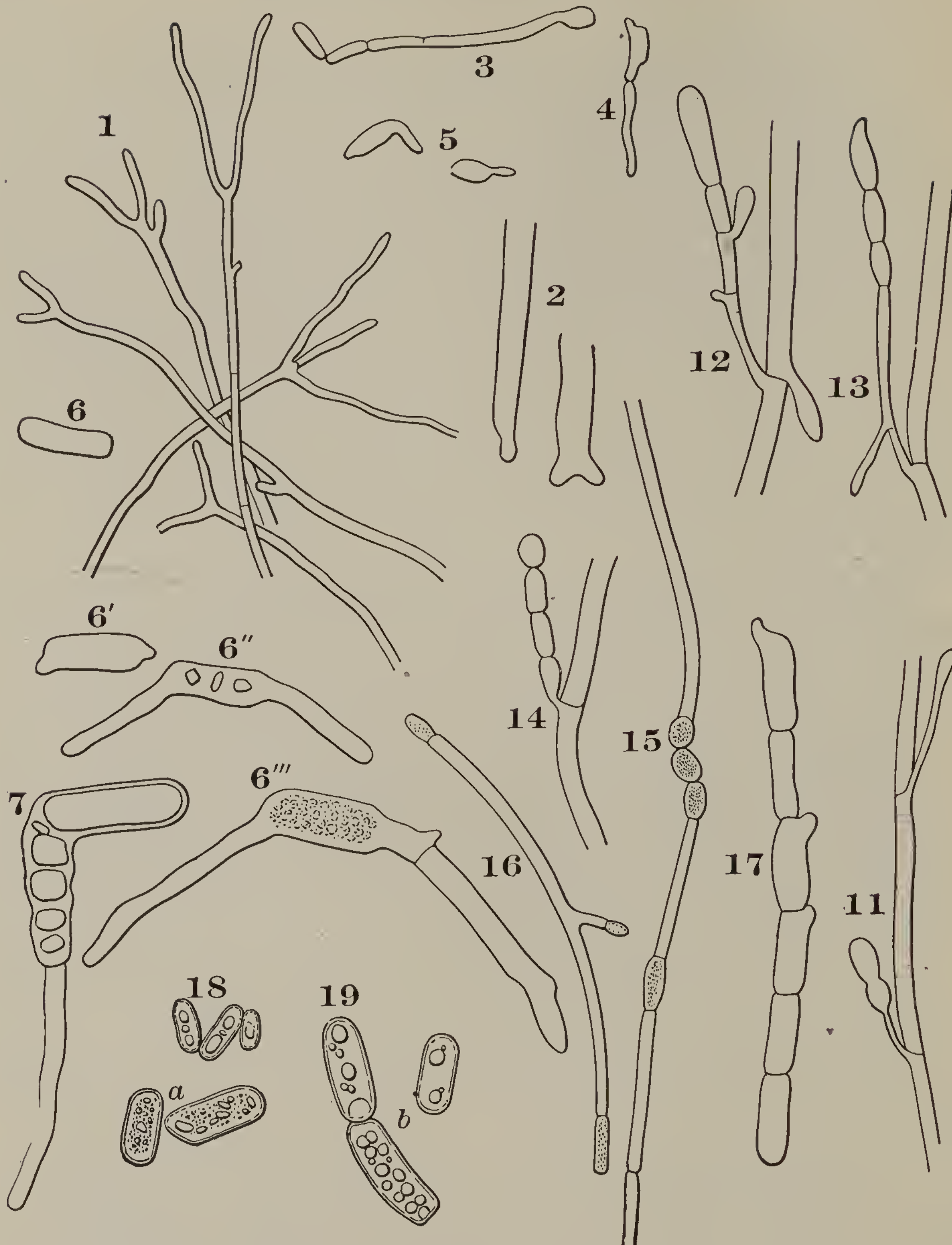


Fig. 35.—*Oospora* (*Oidium*) *lactis* (after Hansen) see p. 104, Jörgensen.

1. Hyphæ with forked partitions; 2, two ends of hyphæ—one with forked partition, the other with commencement of development of a spherical link; 3–7, germinating conidia; 6–6‴, germination of a conidium, sown in hopped beer-wort in Ranvier's chamber, and represented at several stages; at each end germ tubes have developed; after nine hours (6‴) these have formed transverse septa and the first indications of branchings; 11–14, abnormal forms; 15, 16, hyphæ with interstitial cells, filled with plasma; 17, chain of germinating conidia; 18, conidia which have lain for some time in a sugar-solution; the contents show globules of oil; 19, old conidia.

State the age of the culture. These cider tubes are then of the proper age from which to make inoculations.

5. *Pure cultures* of the two remaining molds will be found in tumblers marked "Laboratory Cultures."

Always leave such cultures in the place assigned, after using.

Make cultures of each of the four molds as follows:

(*a*) Agar slant (for method see Exercise 15).

(*b*) Agar plate (giant colony). Use Roux culture flask for *Rhizopus nigricans* (see pp. 2, 60).

(*c*) Cider or wort (test-tube culture).* For method of inoculation of *c* and *d* see pp. 58, 59.

(*d*) Gelatin stab (test-tube culture). Keep all gelatin test-tube cultures *in cold-water bath* or in a cool place (15° to 20° C).

(*e*) Adhesion or moist-chamber culture. (See pp. 76–79.) Prepare these cultures from the *freshly inoculated* cider tubes.

6. *Make drawing of spores of each mold* from adhesion or moist-chamber culture *as soon as preparation is made.* Measure the spores and record the limits of size.

7. Draw the twenty-four hour cultures each of *a*, *b*, *c*, *d*, and *e* and label in detail. Measure the spores which have germinated in *e*, and record the diameter and length of the mycelium.

8. Make drawings of branched mycelium and several stages of development of fruiting bodies from a glycerin mount. This mount is most easily prepared from an agar plate colony.

9. Make drawings of all cultures as soon as a *marked* development is seen over that of the preceding drawing. Three drawings of each culture should be sufficient.

10. Measure giant colony of each mold every day and record the measurements. What is noted of the comparative rate of growth?

All drawings must be made directly on charts or in note-

* Cider cultures have already been made of the two molds isolated from mixed culture.

books as assigned. *Describe the drawings at the time they are made.* Descriptions are to be recorded in ink; use a 6H pencil for drawings.

This outline or some modification of it may be employed for the various species of molds.

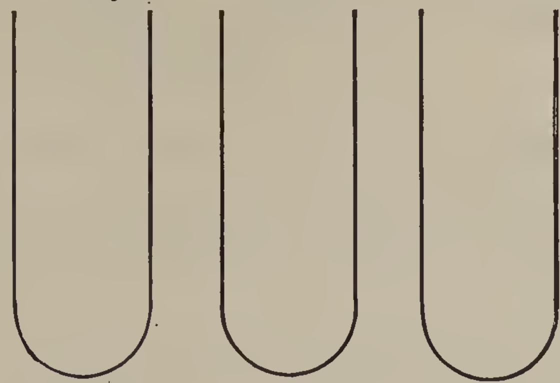
MOLDS

Name of student	Desk No.
Name of organism	Isolated from
Method of isolation	
Occurrence	
Importance	
Spore	
Stages of germination	Drawn from..........preparation
Mycelium	Drawn from..........preparation
Method of reproduction	Drawn from..........preparation
Total organism	Drawn from..........preparation

Note: Mold, yeast and bacteria charts (pp. 108–109, 120–121, 133–136) may be procured from the Campus Press, East Lansing, Mich.

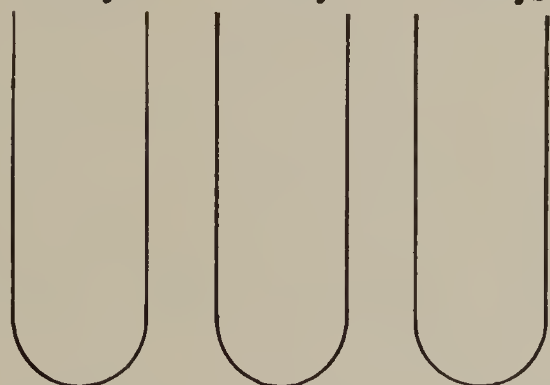
1 day ......days .....days

Cider or wort
culture
Titre...........
Incubated at
..........C.



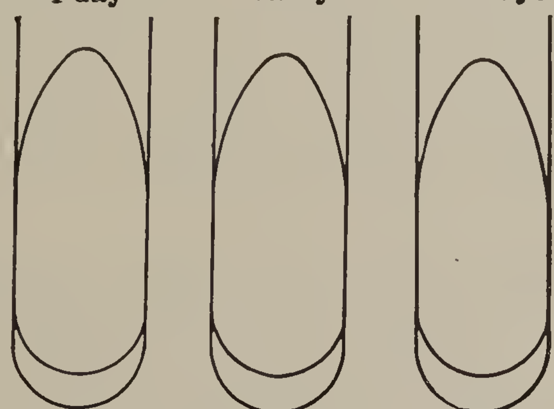
1 day ......days .....days

Nutrient gelatin
stab
Titre...........
Incubated at
.............C.



1 day ......days .....days

Agar streak
Titre...........
Incubated at
...........° C.



Age of colony

Size of colony

Surface elevation

Gelatin or agar
colony
Titre...........
Incubated at
...........° C.

EXERCISE 38. TO DEMONSTRATE QUALITY OF TECHNIC

Apparatus. 3 tubes of sterile agar; 3 sterile Petri dishes; platinum loop.

Method. **1.** Melt the tubes of agar in steam and keep at 45° C.

2. Number tubes 1, 2, and 3.

3. Number plates to correspond.

4. Sterilize the platinum loop in the flame.

5. As soon as the needle is cool, transfer two loopfuls of the agar from tube No. 1 to tube No. 2.

6. Sterilize the needle.

7. Transfer two loopfuls from tube No. 2 to tube No. 3.

8. Pour each tube of agar into the plate with corresponding number.

9. Incubate at room temperature.

10. Examine after one day, six days, seven days, etc.

11. If your plates show no growth in six days, show them to the instructor, otherwise repeat the experiment.

12. Keep the plates to see how long they will remain sterile.

13. Why should the desk be dampened with mercuric chloride? Explain why the mouth of the test-tube and the edge of the Petri dish should be flamed.

14. Would an open window in the laboratory influence the results? At what time of the year is this most likely to influence the results?

15. Why should the tube of agar be dried on the outside before pouring the plate? What type of microorganisms may occur in ineffectively sterilized glassware?

REFERENCE

Lafar: Technical Mycology, Vol. II, Part II, pp. 353-361.

EXERCISE 39. TO DEMONSTRATE THE PATHOGENIC NATURE OF MOLDS

Apparatus. One deep culture dish; one perfect fruit the same as that from which the mold was isolated, or any fruit which is the natural habitat of the mold.

Cultures. Pure culture of a mold isolated from a fruit.

Method. **1.** Make small circles on opposite sides of the fruit with the wax pencil.

2. Puncture the center of one circle with a sterile platinum needle.

3. Then with needle contaminated with the mold spores inoculate the circle on the opposite side by puncturing as in 2.

4. Place at about 25° C. and observe from day to day for two weeks. Cut the fruit through the points of inoculation and note results.

5. How do fruits usually become contaminated with molds? What preventive measures would you suggest?

6. What is a *perfect* fruit from the bacteriological standpoint? From the horticultural standpoint? May these view-points differ? If so, how? What other fruits would be susceptible theoretically to the mold you used? Why?

What other types of microorganisms may be pathogenic to fruits?

7. State in full the results obtained, with any conclusions that may be drawn, and point out the practical application which may be made.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 602, 612, 614, and 625.

SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases, Vol. I, p. 202, Plates 29, 30 and 31.

SMITH, ERWIN F.: Bacteria in Relation to Plant Diseases, Vol. II, Fig. 13, pp. 60 and 174–181.

YEASTS

The so-called yeasts are divided into true yeasts "*Saccharomycetes*" (wild and cultivated), and pseudo-yeasts or false yeasts, "*Torulæ*" and "*Mycodermata*."

By true yeasts are meant those which usually produce alcoholic fermentation (*Sacch. membranæfaciens* is an exception), and which are able to form endospores.

Pseudo-yeasts do not form endospores and produce little or no alcoholic fermentation.

Sacch. cerevisiæ, the yeast used in the manufacture of beers and in bread-making, is a good example of the cultivated yeast.

Sacch. apiculatus and *Sacch. ellipsoideus* are examples of yeasts which are involved in the making of wines. (The latter is cultivated and pure cultures used to some extent.)

Torula rosea is an example of the pseudo-yeast. These look like true yeasts, reproduce by budding, but seldom produce alcoholic fermentation.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 59–76, 443, 506, 508–512, 515, 524, 529, 535, 538, 551.

Klöcker: Fermentation Studies, pp. 205, 249, 289, 296.

EXERCISE 40. TO ISOLATE A PURE CULTURE OF SACCHAROMYCES CEREVISIÆ AND TO STUDY THE FLORA OF A COMPRESSED YEAST CAKE

Apparatus. Cover-glasses; concave slide; sterile Esmarch dishes; potato knife; platinum needles; Bunsen burner; sterile pipette; three tubes of sterile dextrose agar; iodin solution; methylen blue (0.0001% aqueous solution); three sterile Petri dishes.

Culture. Fresh compressed yeast cake.

A. Isolation of Saccharomyces Cerevisiæ

Method. **1.** Sterilize the potato knife in the flame of the Bunsen burner.

2. As soon as cool, cut a piece off the yeast cake.

3. Make three dilution plates in dextrose agar immediately from this freshly cut surface. *Use the straight needle* and transfer only a *very minute* quantity of the yeast. Distribute well with the platinum needle. Use the *straight needle* for making dilutions *in all cases.*

4. When the colony develops (three to six days) examine under objective No. 3, ocular No. 1, inverting the plate for this purpose.

The individual cells of most yeast colonies may be seen under objective No. 3, while individual bacteria can seldom be distinguished in the colony at this low magnification.

5. When you have located a yeast colony make a hanging drop from it in water and determine the shape of the individual yeast cells.

6. If they have the shape and size of *Sacch. cerevisiæ* (see Marshall, p. 72), inoculate a tube of wort from this colony.

7. Study this yeast according to directions in Exercise 42.

B. Study of Flora of Compressed Yeast Cake

Method. **1.** After preparing plates, place the yeast cake in a sterile Esmarch dish.

2. Add 1 c.c. of boiled water, using a sterile pipette.

3. From the freshly cut surface, prepare a hanging drop of the yeast in water, adding a loopful of iodin solution to it. Yeast cells will be unstained, while starch grains become blue.

4. Repeat every seven days.

5. Is the cake made up mostly of starch grains or yeast cells? What is the purpose of the starch in the yeast cake? Do the starch grains remain intact or do they disappear? Explain. What kinds of starch are used?

6. Draw and measure the starch grains. A drawing of the individual yeast cell may be made from this mount.

7. Prepare a second hanging drop of yeast in water from the fresh cake.

8. Stain by adding a loopful of 0.0001% aqueous methylen blue. Dead yeast cells are stained blue, while the living cells remain unstained.

9. Count the number of living and dead cells in each of several fields. Estimate the per cent of living and dead yeast cells.

10. Repeat every seven days until all the yeast cells are dead.

11. How long does this take? What factors influence the death rate? Do other microorganisms enter? If so, what types? Why? From what source? Do they influence the value of the yeast cake? How?

12. Each time you record the percentage of living and dead cells, note the macroscopical appearance of the cake. Also note the presence of new microorganisms, consistency of the cake, odor, and color.

13. Record the results of this experiment in tabulated form, and state any conclusions that may be drawn or practical application to be made.

REFERENCES

Iago, Wm. and Jago, Wm. C.: The Technology of Breadmaking. (1911), pp. 235–239.

Conn: Yeasts, Molds and Bacteria, pp. 56–99.

Schneider: Bacteriological Methods in Food and Drugs Laboratories. (1915). Plate I, Figs. 2, 3 and 4.

EXERCISE 41. APPARATUS AND METHODS FOR THE STUDY OF GASEOUS FERMENTATION

Various forms of yeasts, bacteria and other micro-organisms have the ability to ferment carbohydrate, nitrogenous, and other food substances with the liberation of gas.

A. Smith's Fermentation Tube

Theobald Smith (1893) introduced the use of a special tube for studying fermentation and gas production, and now Smith's fermentation tube is in general use in this and other countries.

Its value lies in the fact that it is a simple apparatus, yet it allows not only of testing the relative fermentative powers of different species of microorganisms or of different strains of the same species, but of determining the gases produced qualitatively and their relative proportions quantitatively to some extent.

Apparatus. Smith fermentation tubes; gasometer; nutrient carbohydrate broth (or any desired solution); platinum needles.

Culture. Culture of the organism to be tested.

Method. **1.** The carbohydrate broth (or other liquid medium) is placed in the fermentation tubes, filling the long arm by carefully tilting. The bulb should be filled with the liquid only to the extent that air will not enter the long arm upon slightly tilting. The tube should not be filled so full that the bulb will not contain all of the liquid in the long arm.

2. Sterilize. Carbohydrate broths are sterilized by the intermittent method.

3. Inoculate fermentation tubes of the desired medium with the organism to be tested, using a loop or straight needle.

4. Incubate at optimum temperature.

5. Examine in twenty-four hours for gas production, and mark the level of the liquid in the long arm of the

fermentation tube each day if gas is being formed. (If the level is *higher* than it was the previous day, the gas (CO_2) is being *absorbed*. Do not allow this absorption to proceed further, but test the gas present for CO_2 and H_2).

6. Measure and record the amount of gas by means of a gasometer (see illustration). The total amount is not

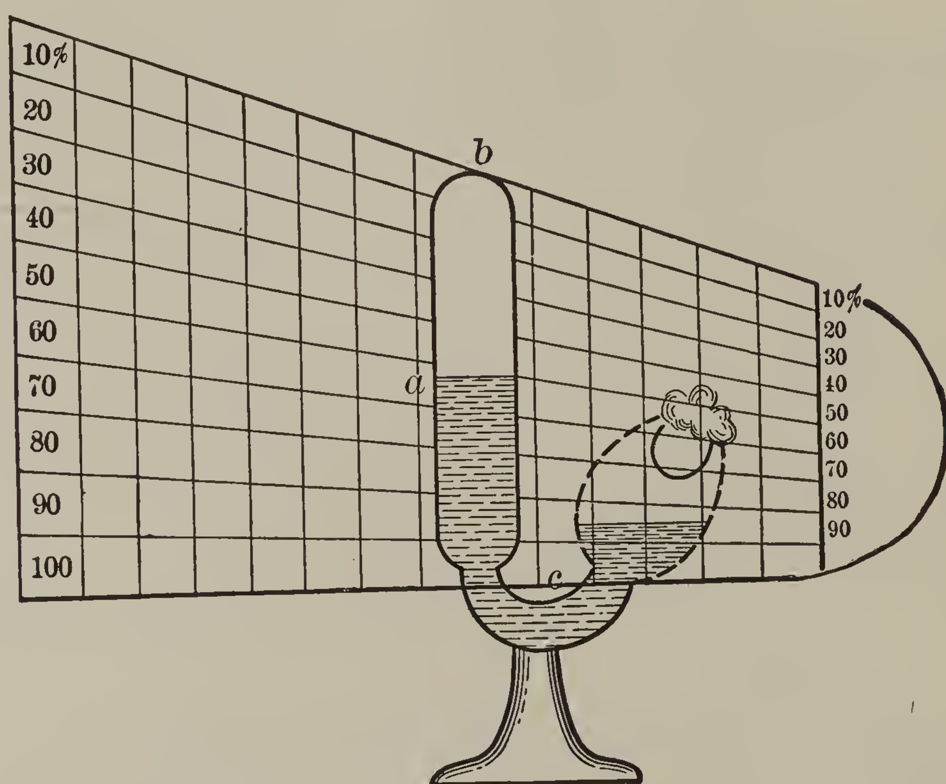


Fig. 36.—Smith's Fermentation Tube Showing use of Frost's Gasometer.

exact quantitatively, as some gas is given off from the open arm of the fermentation tube.

7. When the maximum amount of gas is formed, test the gas for CO_2 and other gases as follows:

Fill the short arm of the fermentation tube with 10% NaOH. Place the thumb over the mouth of the tube and shake vigorously, so that the gas contained in the long arm comes in contact with the NaOH.

$$2NaOH + CO_2 = Na_2CO_3 + H_2O.$$

Sodium carbonate and water are formed, leaving the other gases free.

Collect in the long arm of the tube all the gases remaining. Remove the thumb. The difference in the per cent of gas before and after treating with the NaOH equals the per cent of CO_2 which was present.

8. Place the thumb over the mouth of the tube and *collect all remaining gas in the short arm.* Light a match, remove thumb and *immediately* touch off the remaining gas. If H_2 is present the typical reaction occurs. Other gases are often present, but in too small amounts to allow of testing.

10. Record the relative proportions of CO_2 and H_2 formed.

B. Durham's Fermentation Tube

Durham's fermentation tube is simply an ordinary test tube containing a sugar broth, in which a smaller test tube, inverted, has been placed before sterilization.

This apparatus possesses some advantages over the Smith fermentation tube if only the presence of gas production is to be noted, as the tubes are more easily cleaned, sterilized and handled.

The amount of gas may be roughly estimated, but the kind of gas may not be determined by the use of this apparatus.

EXERCISE 42. THE STUDY OF YEASTS

The object of this exercise is to demonstrate how to differentiate yeasts by microscopical and cultural methods.

Apparatus. Clean cover-glasses; three clean concave slides; five clean fermentation tubes; one tube sterile 2% dextrose broth;* one tube sterile 2% lactose broth;

* The sugar and glycerin broths are furnished by the laboratory.

one tube sterile 2% saccharose broth; one tube sterile 2% glycerin broth; one tube sterile nutrient broth; three tubes sterile cider; three tubes sterile gelatin; four tubes sterile dextrose agar; gasometer; 10% NaOH; litmus solution.

Cultures. *Saccharomyces cerevisiæ;* Saccharomyces apiculatus; Torula rosea; Mycoderma cucumerina.*

Method. 1. Fill one fermentation tube with each broth. Sterilize at fifteen pounds pressure for twenty minutes in autoclav or by heating one-half hour in the steam on three consecutive days.

2. *Make cultures of each yeast in*

(*a*) Cider.
(*b*) Gelatin (stab culture).
(*c*) Dextrose agar slant.
(*d*) Dextrose agar plate (giant colony).†
(*e*) Lindner's concave slide culture (p. 81).

3. Make cultures of *Saccharomyces cerevisiæ only*, in fermentation tubes of

(*a*) Plain broth—control—(without carbohydrate).
(*b*) 2% dextrose broth.
(*c*) 2% lactose broth.
(*d*) 2% saccharose broth.
(*e*) 2% glycerin broth.
(*f*) 2% soluble starch broth.

4. Prepare an *adhesion culture* from *freshly inoculated* wort culture of the yeast (see p. 76).

5. Examine microscopically *immediately after preparation* and draw single cells and cells in various stages of budding (gemmation); show interior structure of cells.

6. *Examine all cultures after twenty-four hours*, and make

* *Saccharomyces cerevisiæ* has been previously isolated from a fresh cake of Fleischmann's compressed yeast. (See Exercise 40.)

† Only one plate is necessary. All yeasts may be grown on one plate. Use dextrose agar.

drawings of (*a*), (*b*), (*c*), (*d*), and (*e*) under **2**, place as indicated on chart, and label correctly.

7. Describe all gelatin and agar cultures according to the descriptive chart of the American Society of Bacteriologists (p. 132). In describing the wort culture use the descriptive chart terms under the heading "Nutrient broth."

8. If any gas has formed in the fermentation tubes mark the level of the liquid in the long arm with a wax pencil and record the percentage of gas, using the gasometer. Test for acid, and alcohol (odor).

9. Test quantitatively and qualitatively for gas in the fermentation tubes. (See Exercise 41, p. 115).

10. What is the ratio of the CO_2 to H_2 and other gases? Is this ratio constant for all fermentations? For one organism? Why? Do all organisms cause fermentation? Why? What causes fermentation?

11. Examine adhesion cultures after forty-eight hours and seventy-two hours and make drawing of colony formation.

12. Study the fourteen to twenty-day old wort cultures in hanging drop for endospores. When do these form? Why?

REFERENCES

Hansen, E.: Practical Studies in Fermentation, pp. 215–217.

Lafar: Technical Mycology, Vol. II, Part II, pp. 394–406, 430–436.

Eyre: Bacteriological Technic. Second Ed., p. 7.

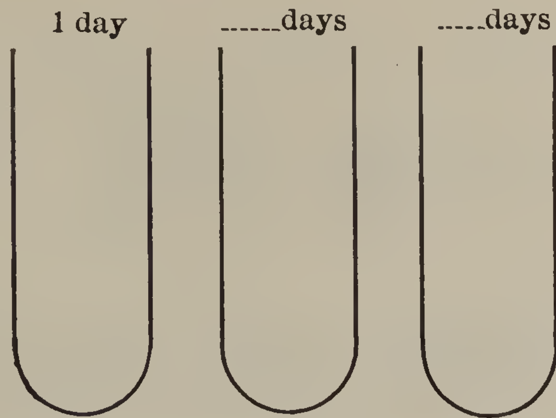
YEASTS

Name of student	Desk No.
Name of organism	Isolated from
Method of isolation	
Occurrence	
Importance	
Total organism	Drawn from..........preparation
Stages of budding	Drawn from..........preparation
Method of reproduction	Drawn from..........preparation
Spore	

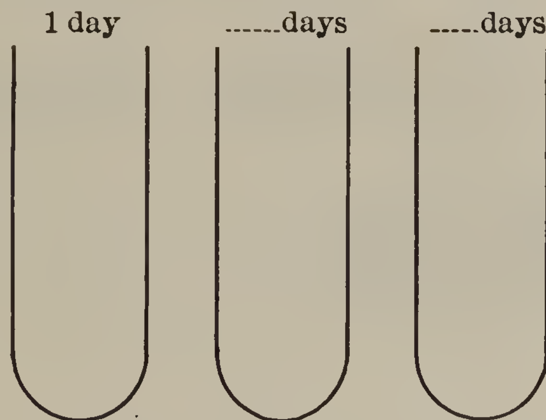
Fermentations

% Of gas in	Control	Dextrose	Lactose	Saccharose	Glycerin
24 hours					
48 hours					
3 days					
5 days					
Total gas production					
Ratio of CO_2:H_2 and other gases					
Alcohol (odor)					
Acid					
Growth in closed arm					

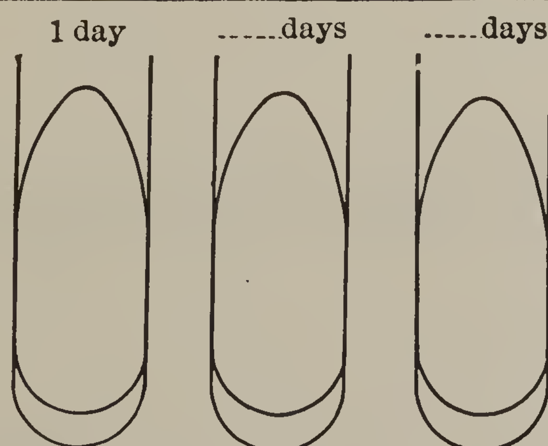
Cider or wort culture
Titre............
Incubated at
............° C.



Nutrient gelatin stab
Titre.............
Incubated at
............° C.



Agar streak
Titre.............
Incubated at
............° C.

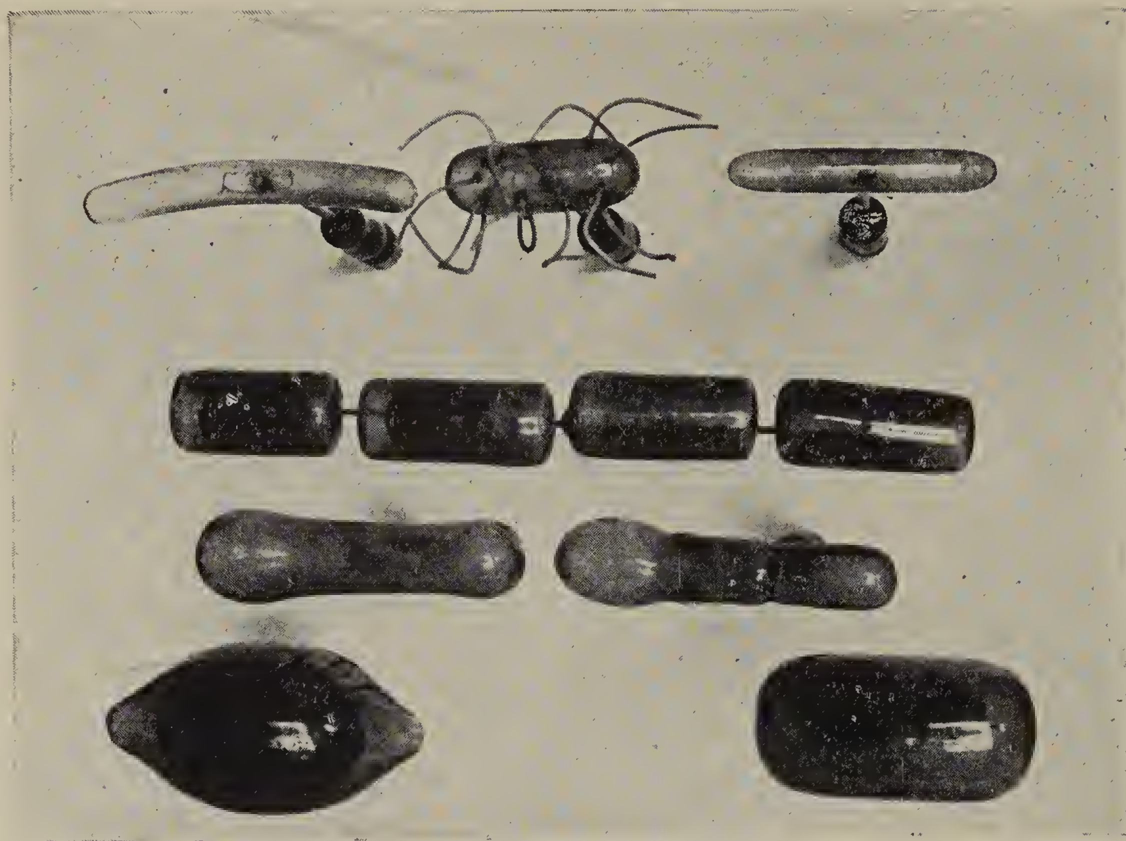


Age of colony

Size of colony

Surface elevation

Gelatin or agar colony
Titre............
Incubated at
............° C.



EXERCISE 43. THE STUDY OF BACTERIA

Studies will be made of ten bacteria representing the different morphological types. These are to be identified by morphological and cultural characteristics.

Pure cultures of these organisms will be found on each desk in the tumblers marked "Laboratory cultures." Always return laboratory cultures to these tumblers immediately after using.

DANGER. Some of these organisms are pathogenic. If you do not handle them with care and according to directions you endanger not only yourself, but all working in the laboratory. Do not be careless. Handle all organisms as if they were pathogenic. This is a good habit; get it immediately. (See "Care of Cultures," pp. 45–46.) The instructor will designate which organisms are pathogenic.

Apparatus. Clean cover-glasses; clean concave slides; clean plain slides; ten agar slants; ten tubes sterile agar for plates; ten tubes nutrient broth; ten tubes nutrient gelatin; ten tubes litmus milk; ten tubes glycerin potato; ten tubes Dunham's solution; ten tubes nitrate peptone solution; four fermentation tubes of plain broth; four fermentation tubes of dextrose broth; four fermentation tubes of lactose broth; four fermentation tubes of saccharose broth; centimeter scale; gasometer; lead acetate

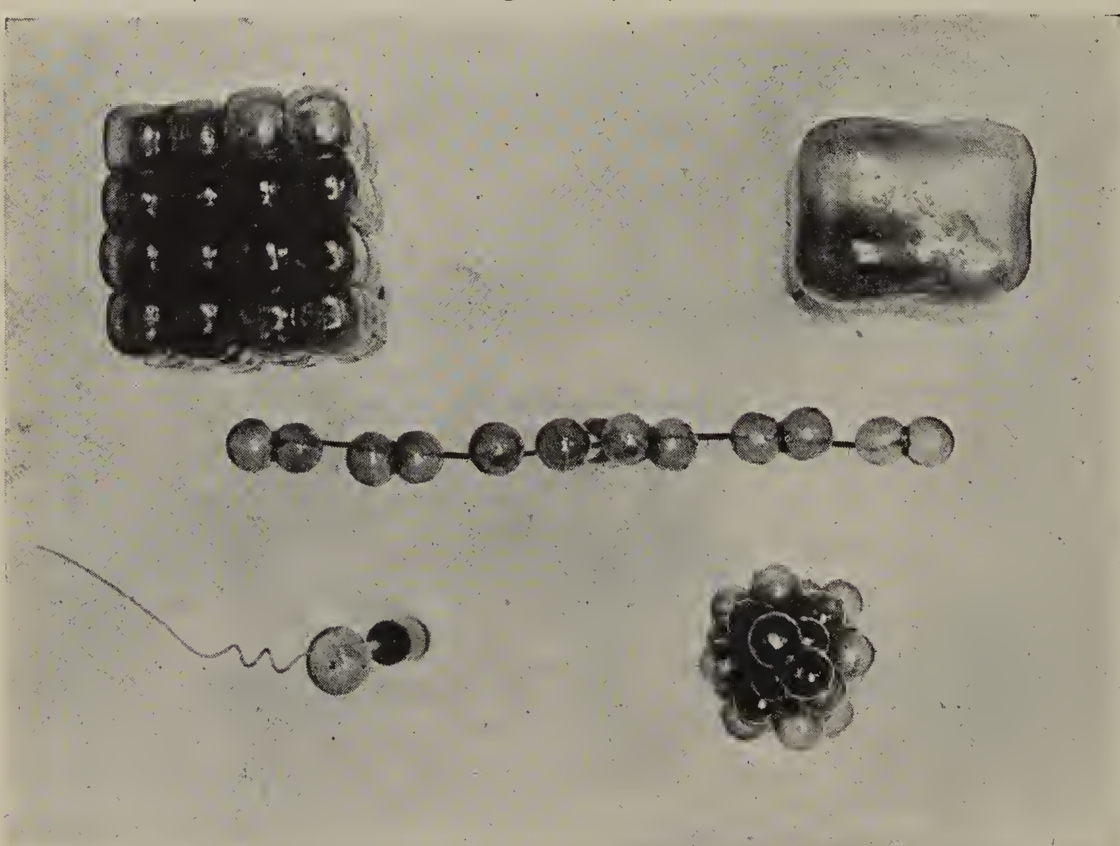
---

DESCRIPTION OF PLATE V

I. 1, *Bact. tuberculosis;* 2, *B. typhosus;* 3, *Bact. lepræ;* 4, *Bact. anthracis* (strepto-bacterium, two with spores); 5, *Bact. diphtheriæ* (club-shaped); 6, anthrax spore, germinating (polar); 7, *B. amylobacter* (clostridium); 8, *Streptococcus pneumoniæ* (diplococcus with capsule).

II. 1, *B. subtilis* (strepto-bacillus, peritrichous flagella, one with spore); 2, *B. subtilis* (peritrichous flagella); 3, formation of a new filament from a germinating spore; 4, spore of *B. subtilis;* 5, germinating spore of *B. subtilis* (equatorial); 6, beginning germination.

III. 1, *Spirillum volutans* (Cohn) with lophotrichous flagella (chain of three); 2, *Sp. volutans*, single cell; 3, *Microspira comma*, monotrichous flagellum; 4, *Spirocheta obermeieri*.



IV. 1, *Sarcina lutea;* 2, *Micrococcus tetragenus* with capsule; 3, streptococcus; 4, planococcus; 5, staphylococcus.

paper; aqueous-alcoholic fuchsin and methylen blue; mordant for flagella stain; Lugol's iodin solution; anilin-

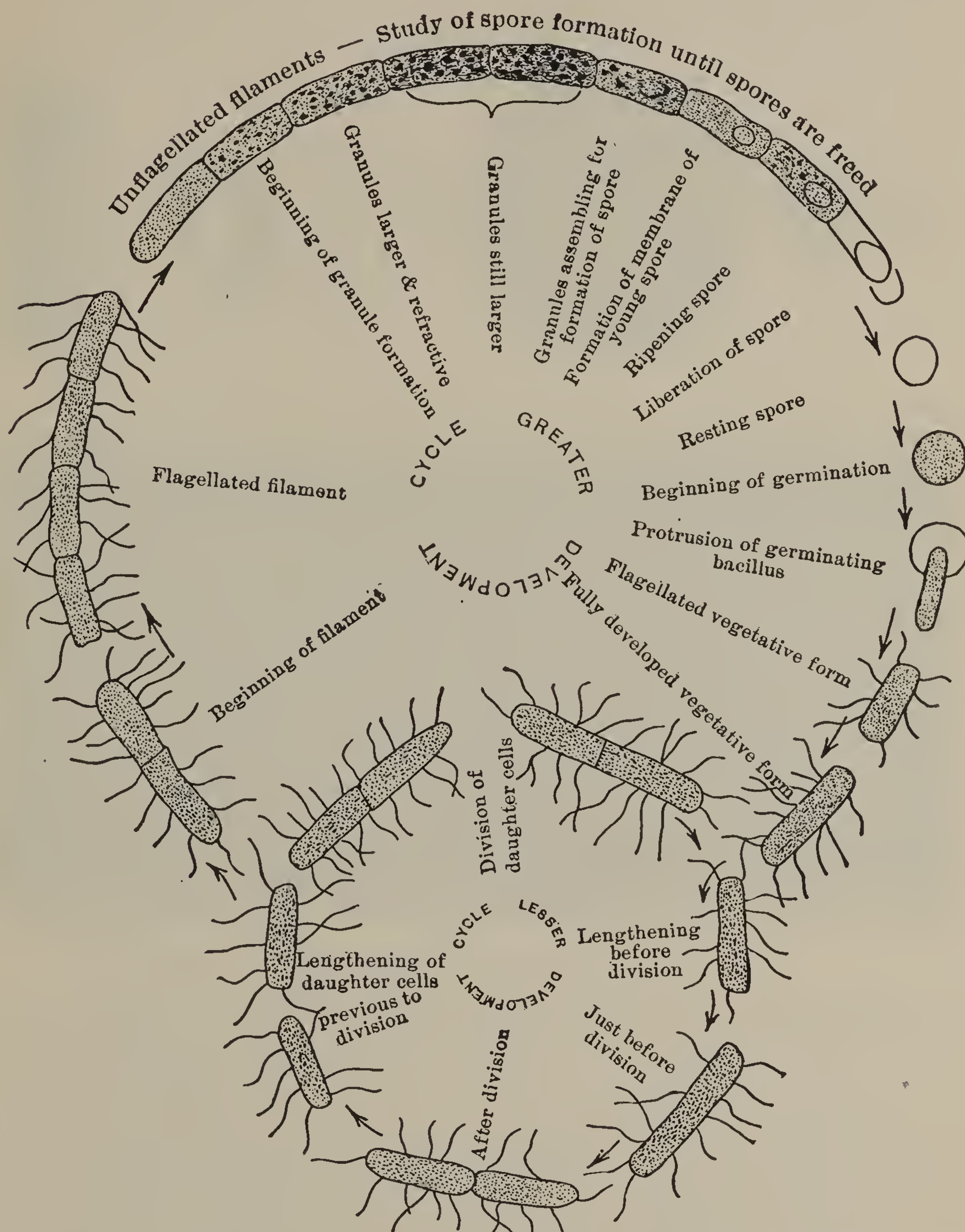


FIG. 37.—Cycle of Development of Bacterial Cell. (Adapted from Fuhrmann's Technische Mykologie.)

water gentian violet; carbol-fuchsin; acetic acid-alcohol for decolorizing spore stain; indol test solutions; nitrate test solutions; ammonia test solutions.

Method. **1.** Make an agar slant culture of each organism and incubate each at its optimum temperature. (Instructor will designate the optimum temperature of each.)

FIG. 38.—Comparative Sizes of Bacteria.

1, *Micrococcus progrediens*, 0.15μ; 2, *Micrococcus ureæ*, 1–1.5μ; 3, *Sarcina maxima*, 4μ; 4, *Thiophysa volutans* (sulphur bacteria), 7–18μ; 5, influenza bacillus, 4.2$\times$0.4μ; 6, methane bacillus, 5$\times$0.4μ; 7, *Urobacillus duclauxii* (Miquel), 2–10$\times$0.6–0.8μ; 8, *Bacillus nitri* (Ambroz), 3–8$\times$2–3μ; 9, *Beggiatoa alba* (sulphur bacteria), 2.9–5.8$\times$2.8–2.9μ; 10, *Chromatium okenii*, (sulphur bacteria), 10–15$\times$5μ; 11, *Beggiatoa mirabilis* (sulphur bacteria), 20–25$\times$40–50μ. (From Fuhrmann's Technische Mykologie.)

2. Draw and describe twenty-four-hour old agar slant cultures, then examine microscopically in hanging drop to determine the morphology, size, grouping or arrangement,

motility, spores. Use ocular No. 1 and objective No. 7. The greatest motility will be observed in organisms growing in the condensation water at the base of the slant.

3. Draw the total organism and record the presence or absence of motility. *Describe all cultures at the time the drawings are made* of each, following the terminology of the "Descriptive Chart of the American Society of Bacteriologists," p. 132.

4. Use drawing pencil for making drawings and ink for recording descriptions.

Any descriptive terms may be added which will aid in identifying organisms, but descriptive chart terms must be followed as closely as possible, otherwise *drawings will not be accepted.*

Always state the *age of the culture*, the *temperature at which the organism is grown*, the *medium* upon which it is cultivated and the *titre of the medium.*

Use one chart for each organism.

5. When the agar slant culture of each organism shows good growth, make inoculations from this culture into the following media:

Agar plate (see below for method).
Gelatin plate (see step 6, below).
Nutrient broth.
Nutrient gelatin (stab culture).
Litmus milk.
Glycerin potato.
Dunham's solution.
Nitrate peptone solution.
Plain broth fermentation tube (control).
Dextrose broth fermentation tube.
Lactose broth fermentation tube.
Saccharose broth fermentation tube.

6. In preparing *agar plate from bacterial cultures*, proceed as follows: Inoculate a tube of nutrient broth *lightly*, using

the *straight* needle. Then, still using the straight needle, from the freshly prepared broth culture, inoculate *lightly* one tube of melted agar (at 40° to 50° C.) and pour into a sterile Petri dish. If the organism shows only a slight growth on the stock culture, transfer directly to melted agar.

7. Moisten a strip of lead acetate paper and insert with cotton plug in tube of Dunham's solution. Blackening of this paper shows the formation of H_2S.

Between what substances does a chemical reaction take place? What are the resulting products?

8. Draw and describe twenty-four-hour cultures of the first four bacteria in all media. If at any time presence of growth is doubtful, compare with a tube of sterile medium. In the absence of growth, reinoculate.

9. Record *macroscopical* changes *only*, in litmus milk; and in fermentation tubes note only, the place of growth, presence and percentage of gas; also the formation of H_2S in Dunham's solution.

10. Make a *permanent stained preparation of each organism* (following directions under Exercise 28). *Young* (twenty-four to forty-eight hour) *cultures must be used.* Use either aqueous-alcoholic fuchsin or aqueous-alcoholic methylen blue.

11. Make a *flagella stain* of the largest motile organism among your cultures.

It is *absolutely necessary* that a *young* (eighteen to twenty-four hour, *not older*) *culture be used* for this purpose. Follow the directions under Exercise 31.

12. Make further drawings and descriptions from day to day *if any change in the growth from that of the preceding day is observed. Three* drawings of a culture will be sufficient. *Endeavor to illustrate typical growth by careful drawings.*

13. State whether the agar plate colony described is a surface or a subsurface colony. How do these two types of colonies differ? Why?

14. Note the presence of condensation water, whether a small or large amount is present. How does this affect colony development?

15. *Draw and measure a typical surface and subsurface colony produced by each organism.*

The form and size often vary with the physical condition under which the colony grows or with physiological conditions, i.e., the proximity of colonies producing poisonous metabolic products.

16. Examine cultures three to six days old in hanging drop for presence of spores. Spores may be seen *free* or enclosed in the bacterial cells. They are easily distinguished by their refractivity. Ordinary anilin dyes will not stain them.

17. Make a *contrast spore stain* of a spore-forming organism. (For method see Exercise 29.)

Draw and describe *only the mature cultures* of the last six organisms (five to eight days old).

18. Make the indol, nitrate and ammonia tests also on the *mature* cultures.

19. In fermentation tube cultures note and record the oxygen requirements of each organism; total per cent of gas; ratio of $CO_2 : H_2$ and other gases.

20. Test each organism after seven days for indol, nitrate and ammonia production. The culture in Dunham's peptone solution is tested for indol (for method see Exercise 44).

Divide the nitrate peptone solution culture into two parts; test one for nitrates, the other for ammonia (for method see Exercise 45).

21. Prepare permanent stained preparations of one Gram-positive and one Gram-negative organism.

22. Making use of morphological and cultural characteristics ascertained microscopically and by the various cultural tests, identify each organism, using Chester's Manual of Determinative Bacteriology for tracing out

the genus and species. Other valuable reference texts are:

CONN, ESTEN and STOCKING: Classification of Dairy Bacteria.
NOVY: Laboratory Manual of Bacteriology.
JORDAN: General Bacteriology.

EXERCISE 44. EHRLICH'S METHOD OF TESTING INDOL PRODUCTION

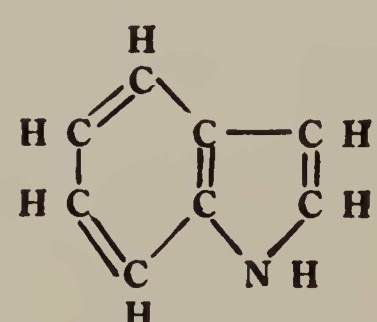
The purpose of the exercise is to test the power of an organism to produce indol from peptone.

Cultures for comparison should be of the same age and grown in the same kind of medium. Some peptones contain a trace of indol and, to avoid all possibility of mistake when testing for indol, a control tube of sterile medium should be used at the same time. This reaction is characteristic for indol or for methyl indol (skatol).

There are other tests for indol, but this one is by far the most delicate. The Salkowski-Kitasato test (conc. H_2SO_4 and $NaNO_2$) will detect indol in a dilution of only 1 : 100,000, while Ehrlich's test will give a reaction in a dilution ten times greater, or 1 : 1,000,000.

Indol is one of the most important of protein decomposition products. It is noted for its intense fecal odor. However, in highly dilute solutions it has the odor of orange-blossoms, hence is used extensively in perfumery. The jessamine blossom contains indol and has its odor.

Indol has the following graphic formula:



According to Emil Fischer, the reaction of Ehrlich's test, produces, by means of the oxidizing action of the potas-

sium persulphate, a condensation of two molecules of indol with the aldehyde group of the para-dimethyl-amido-benzaldehyde, water splitting off.

Apparatus. Solutions I and II for Ehrlich's test for indol;* two clean 5 c.c. pipettes.

Culture. Dunham's peptone solution or broth culture of the organism to be tested.

Method. 1. To about 10 c.c. of the liquid culture add 5 c.c. of solution I, then 5 c.c. of solution II.

2. Shake the mixture. The reaction may be accelerated by heating. The presence of indol is indicated in a few minutes by a red color which increases in intensity with time. For standard comparisons, five minutes is taken as the maximum time limit.

REFERENCES

Boehme, A.: Die Anwendung der Ehrlichschen Indol-reaktion für Bakteriologische Zwecke. Cent. f. Bakt. Orig. Bd. 40 (1906), pp. 129–133.

Besson: Practical Bacteriology, Microbiology and Serum Therapy (1913), p. 374.

Löhnis: Laboratory Methods in Agricultural Bacteriology (1913), p. 42.

Lewis, F. C.: On the detection and estimation of bacterial indol and observations on intercurrent phenomena. Jour. Path. and Bact., Vol. 19 (1915), pp. 429–443.

EXERCISE 45. TESTS FOR THE REDUCTION OF NITRATES

The purpose of the exercise is to test the power of an organism to reduce nitrates.

Apparatus. Sulphanilic acid, nitrite test solution I; *a*-naphthylamin, nitrite test solution II; Nessler's solution; phenolsulphonic acid.

Cultures. Seven-day old nitrate peptone solution cultures grown at 20° to 25° C., or four-day old nitrate peptone solution cultures (pathogenic) grown at 37° C.

Method. (*A*) *For nitrites:* **1.** Add 0.1 c.c. each of solutions I and II to each culture to be tested.

* See Appendix.

2. Repeat with uninoculated control.

3. The development of a red color in ten minutes indicates the presence of nitrites, the intensity of the color depending upon the amount of nitrites present.

(*B*) *For ammonia.* **1.** Add 0.2 c.c. of Nessler's solution to each culture to be tested.

2. Repeat with uninoculated control.

The presence of ammonia is shown by a yellow color or precipitate.

(*C*) *For nitrates unchanged or free nitrogen liberated.* **1.** When either or both of the preceding tests are positive, no further determination need be made, but if negative, then one of two conditions may prevail: (*a*) Either the nitrates have not been changed, or (*b*) they may have been reduced to free nitrogen. To ascertain which is true, it will be necessary to determine the presence or absence of nitrates.

2. Test as follows: (*a*) Evaporate 10 c.c. of each culture and the controls *almost* to dryness in an evaporating dish and add to the residue 1 c.c. of phenolsulphonic acid.

(*b*) Dilute with 10 c.c. distilled water, then add sufficient ammonium hydroxide, diluted 1 : 1 with distilled water, or concentrated potassium hydroxide solution, to make alkaline.

(*c*) Transfer the liquid to a 50 c.c. Nessler tube or graduated cylinder and make up the volume to 50 c.c. with distilled water.

A yellow color shows the presence of nitrates.

BACTERIA

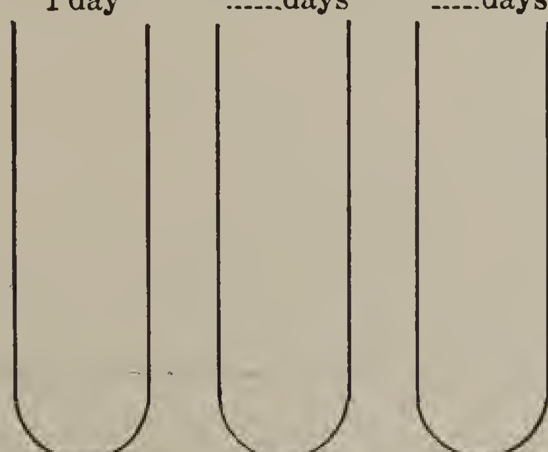
Name of student		Desk No.
Name of organism	Isolated from	
Method of isolation		
Occurrence		
Importance		
Shape of organism	Arrangement	Size
Motility	Flagella	
Method of reproduction	Involution forms	
Spore	Stages of germination	
Aqueous-alcoholic stain	Gram's stain	Acid-fast stain

Agar streak
Titre. . . . .
Incubated at
. . . . ° C.

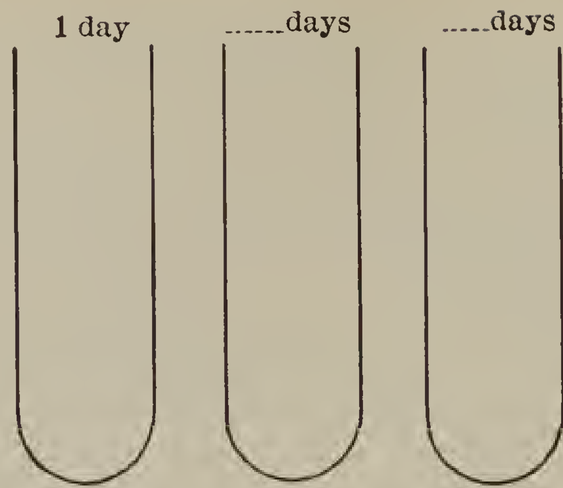
1 day ......days ......days

Gelatin stab
Titre. . . . .
Incubated at
. . . . ° C.

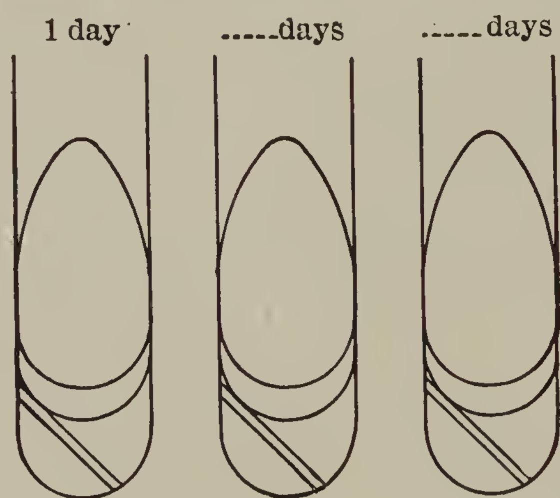
1 day ......days ......days



Broth culture
Titre. . . . .
Incubated at
. . . .° C.



Potato culture
Titre. . . . .
Incubated at
. . . .° C.



Age of agar colony	. . . . . .days	. . . . . .days	. . . . . .days
Size of colony			
Surface elevation			

Agar colony
Titre. . . . .
Incubated at
. . . .° C.

DESCRIPTIVE CHART—SOCIETY OF AMERICAN BACTERIOLOGISTS

Prepared by F. D. CHESTER
F. P. GORHAM
ERWIN F. SMITH
Committee on Methods of Identification of Bacterial Species.

ENDORSED BY THE SOCIETY FOR GENERAL USE AT THE ANNUAL MEETING, DEC. 31, 1907.

GLOSSARY OF TERMS.

AGAR HANGING BLOCK, a small block of nutrient agar cut from a poured plate, and placed on a cover-glass, the surface next the glass having been first touched with a loop from a young fluid culture or with a dilution from the same. It is examined upside down, the same as a hanging drop.

AMEBOID, assuming various shapes like an ameba.

AMORPHOUS, without visible differentiation in structure.

ARBORESCENT, a branched, tree-like growth.

BEADED, in stab or stroke, disjointed or semi-confluent colonies along the line of inoculation.

BRIEF, a few days, a week.

BRITTLE, growth dry, friable under the platinum needle.

BULLATE, growth rising in convex prominences, like a blistered surface.

BUTYROUS, growth of a butter-like consistency.

CHAINS.
Short chains, composed of 2 to 8 elements.
Long chains, composed of more than 8 elements.

CILIATE, having fine, hair-like extensions like cilia.

CLOUDY, said of fluid cultures which do not contain pseudozoogloeæ.

COAGULATION, the separation of casein from whey in milk. This may take place quickly or slowly, and as the result either of the formation of an acid or of a lab ferment.

CONTOURED, an irregular, smoothly undulating surface, like that of a relief map.

CONVEX, surface the segment of a circle, but flattened.

COPROPHYL, dung bacteria.

CORIACEOUS, growth tough, leathery, not yielding to the platinum needle.

CRATERIFORM, round, depressed, due to the liquefaction of the medium.

CRETACEOUS, growth opaque, and white, chalky.

CURLED, composed of parallel chains in wavy strands, as in anthrax colonies.

DIASTASIC ACTION, same as DIASTATIC, conversion of starch into water-soluble substances by diastase.

ECHINULATE, in agar stroke a growth along line of inoculation, with toothed or pointed margins; in stab cultures growth beset with pointed outgrowths.

EFFUSE, growth thin, veily, unusually spreading.

ENTIRE, smooth, having a margin destitute of teeth or notches.

EROSE, border irregularly toothed.

FILAMENTOUS, growth composed of long, irregularly placed or interwoven filaments.

FILIFORM, in stroke or stab cultures a uniform growth along line of inoculation.

FIMBRIATE, border fringed with slender processes, larger than filaments.

FLOCCOSE, growth composed of short curved chains, variously oriented.

FLOCCULENT, said of fluids which contain pseudozoogloeæ, i.e., small adherent masses of bacteria of various shapes and floating in the culture fluid.

FLUORESCENT, having one color by transmitted light and another by reflected light.

GRAM'S STAIN, a method of differential bleaching after gentian violet, methyl violet, etc. The + mark is to be given only when the bacteria are deep blue or remain blue after counterstaining with Bismark brown.

GRUMOSE, clotted.

INFUNDIBULIFORM, form of a funnel or inverted cone.

IRIDESCENT, like mother-of-pearl. The effect of very thin films.

LACERATE, having the margin cut into irregular segments as if torn.

LOBATE, border deeply undulate, producing lobes (see Undulate.)

LONG, many weeks, or months.

MAXIMUM TEMPERATURE, temperature above which growth does not take place.

MEDIUM, several weeks.

MEMBRANOUS, growth thin, coherent, like a membrane.

MINIMUM TEMPERATURE, temperature below which growth does not take place.

MYCELIOID, colonies having the radiately filamentous appearance of mold colonies.

NAPIFORM, liquefaction with the form of a turnip.

NITROGEN REQUIREMENTS, the necessary nitrogenous food. This is determined by adding to *nitrogen-free* media the nitrogen compound to be tested.

OPALESCENT, resembling the color of an opal.

OPTIMUM TEMPERATURE, temperature at which growth is most rapid.

PELLICLE, in fluid bacterial growth either forming a continuous or an interrupted sheet over the fluid.

PEPTONIZED, said of curds dissolved by trypsin.

PERSISTENT, many weeks or months.

PLUMOSE, a fleecy or feathery growth.

PSEUDOZOOGLŒÆ, clumps of bacteria, not dissolving readily in water, arising from imperfect separation, or more or less fusion of the components, but not having the degree of compactness and gelatinization seen in zoogloeæ.

PULVINATE, in the form of a cushion, decidedly convex.

PUNCTIFORM, very minute colonies, at the limit of natural vision.

RAISED, growth thick, with abrupt or terraced edges.

RHIZOID, growth of an irregular branched or root-like character, as in *B. mycoides*.

RING, same as RIM, growth at the upper margin of a liquid culture, adhering more or less closely to the glass.

RAPID, developing in 24 to 48 hours.

REPAND, wavy.

RUGOSE, wrinkled.

SACCATE, liquefaction the shape of an elongated sack, tubular, cylindrical.

SCUM, floating islands of bacteria, an interrupted pellicle or bacterial membrane.

SLOW, requiring 5 or 6 days or more for development.

SHORT, applied to time, a few days, a week.

SPORANGIA, cells containing endospores.

SPREADING, growth extending much beyond the line of inoculation, i.e., several millimeters or more.

STRATIFORM, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.

THERMAL DEATH-POINT, the degree of heat required to kill young fluid cultures of an organism exposed for 10 minutes (in thin-walled test tubes of a diameter not exceeding 20 mm.) in the thermal water-bath. The water must be kept agitated so that the temperature shall be uniform during the exposure.

TRANSIENT, a few days.

TURBID, cloudy with flocculent particles; cloudy plus flocculence.

UMBONATE, having a button-like, raised center.

UNDULATE, border wavy, with shallow sinuses.

VERRUCOSE, growth wart-like, with wart-like prominences.

VERMIFORM-CONTOURED, growth like a mass of worms, or intestinal coils.

VILLOUS, growth beset with hair-like extensions.

VISCID, growth follows the needle when touched and withdrawn, sediment on shaking rises as a coherent swirl.

ZOOGLŒÆ, firm gelatinous masses of bacteria, one of the most typical examples of which is the *Streptococcus mesenterioides* of sugar vats (*Leuconostoc mesenterioides*), the bacterial chains being surrounded by an enormously thickened firm covering, inside of which there may be one or many groups of the bacteria.

NOTES.

(1) For decimal system of group numbers see Table 1. This will be found useful as a quick method of showing close relationships inside the genus, but is not a sufficient characterization of any organism.

(2) The morphological characters shall be determined and described from growths obtained upon at least one solid medium (nutrient agar) and in at least one liquid medium (nutrient broth). Growths at 37° C. shall be in general not older than 24 to 48 hours, and growths at 20° C., not older than 48 to 72 hours. To secure uniformity in cultures, in all cases preliminary cultivation shall be practiced as described in the revised Report of the Committee on Standard Methods of the Laboratory Section of the American Public Health Association, 1905.

(3) The observation of cultural and bio-chemical features shall cover a period of at least 15 days and frequently longer, and shall be made according to the revised Standard Methods above referred to. All media shall be made according to the same Standard Methods.

(4) Gelatin stab cultures shall be held for 6 weeks to determine liquefaction.

(5) Ammonia and indol tests shall be made at end of 10th day, nitrite tests at end of 5th day.

(6) Titrate with N/20 NaOH, using phenolphthalein as an indicator: make titrations at same times from blank. The difference gives the amount of acid produced.

The titration should be done after boiling to drive off any CO_2 present in the culture.

(7) Generic nomenclature shall begin with the year 1872 (Cohn's first important paper).

Species nomenclature shall begin with the year 1880 (Koch's discovery of the poured plate method for the separation of organisms).

(8) Chromogenesis shall be recorded in standard color terms.

TABLE I.

A NUMERICAL SYSTEM OF RECORDING THE SALIENT CHARACTERS OF AN ORGANISM. (GROUP NUMBER.)

100.	Endospores produced.
200	Endospores not produced.
10.	Aerobic (strict).
20.	Facultative anaerobic.
30.	Anaerobic (strict).
1.	Gelatin liquefied.
2.	Gelatin not liquefied.
0.1	Acid and gas from dextrose.
0.2	Acid without gas from dextrose.
0.3	No acid from dextrose.
0.4	No growth with dextrose.
.01	Acid and gas from lactose.
.02	Acid without gas from lactose.
.03	No acid from lactose.
.04	No growth with lactose.
.001	Acid and gas from saccharose.
.002	Acid without gas from saccharose.
.003	No acid from saccharose.
.004	No growth with saccharose.
.0001	Nitrates reduced with evolution of gas.
.0002	Nitrates not reduced.
.0003	Nitrates reduced without gas formation.
.00001	Fluorescent.
.00002	Violet chromogens.
.00003	Blue chromogens.
.00004	Green chromogens.
.00005	Yellow chromogens.
.00006	Orange chromogens.
.00007	Red chromogens.
.00008	Brown chromogens.
.00009	Pink chromogens.
.00000	Non-chromogenic.
.000001	Diastasic action on potato starch, strong.
.000002	Diastasic action on potato starch, feeble.
.000003	Diastasic action on potato starch, absent.
.0000001	Acid and gas from glycerin.
.0000002	Acid without gas from glycerin.
.0000003	No acid from glycerin.
.0000004	No growth with glycerin.

The genus according to the system of Migula is given its proper symbol which precedes the number thus: (7).

BACILLUS COLI (Esch.) Mig.	becomes B.	222.111102
BACILLUS ALCALIGENES Petr.	becomes B.	212.333102
PSEUDOMONAS CAMPESTRIS (Pam.) Sm.	becomes Ps.	211.333151
BACTERIUM SUICIDA Mig.	becomes Bact.	222.232203

(To face page 134.)

Source .... Date of Isolation .... Name .... Group No. (1) ....

DETAILED FEATURES.

NOTE—Underscore required terms. Observe notes and glossary of terms on opposite side of card.

I. MORPHOLOGY (2).

1. Vegetative Cells, Medium used ....
   temp ...., age ....days
   Form, *round, short rods, long rods, short chains, long chains, filaments, commas, short spirals, long spirals, clostridium, cuneate, clavate, curved.*
   Limits of Size ....
   Size of Majority ....
   Ends, *rounded, truncate, concave.*
   Agar Hanging-Block { Orientation (grouping) ....
   Chains (No. of elements) ....
   Short chains, long chains.
   Orientation of Chains, *parallel,* irregular.
2. Sporangia, medium used ....temp ....
   age ....days.
   Form, *elliptical, short rods, spindled, clavate, drumsticks.*
   Limits of Size ....Size of Majority ....
   Agar Hanging-Block { Orientation (grouping) ....
   Chains (No. of elements) ....
   Orientation of Chains, *parallel, irregular.*
   Location of Endospores, *central, polar.*
3. Endospores.
   Form, *round, elliptical, elongated.*
   Limits of Size ....
   Size of Majority ....
   Wall, *thick, thin.*
   Sporangium wall, *adherent, not adherent.*
   Germination, *equatorial, oblique, polar, bipolar, by stretching.*
4. Flagella No ....Attachment, *polar, bipolar, peritrichiate.* How stained ....
5. Capsules, present on ....
6. Zooglœa, Pseudozooglœa.
7. Involution Forms, on ....in....days at....° C.
8. Staining Reactions.
   1: 10 watery fuchsin, gentian violet, carbol fuchsin, Loeffler's alkaline methylen blue.
   Special Stains.
   Gram .... Glycogen ....
   Fat .... Acid fast ....
   Neisser ....

II. CULTURAL FEATURES (3).

1. Agar Stroke.
   Growth, *invisible, scanty, moderate, abundant.*
   Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
   Elevation of growth, *flat, effuse, raised, convex.*
   Luster, *glistening, dull, cretaceous.*
   Topography, *smooth, contoured, rugose, verrucose,*
   Optical Characters, *opaque, translucent, opalascent, iridescent.*
   Chromogenesis (8).
   Odor, *absent, decided, resembling* ....
   Consistency, *slimy, butyrous, viscid, membranous, coriaceous, brittle.*
   Medium, *grayed, browned, reddened, blued, greened.*
2. Potato.
   Growth, *scanty, moderate, abundant, transient, persistent.*
   Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
   Elevation of growth, *flat, effuse, raised, convex.*
   Luster, *glistening, dull, cretaceous.*
   Topography, *smooth, contoured, rugose, verrucose.*
   Chromogenesis (8) .... Pigment in water *insoluble, soluble;* other solvents ....
   Odor, *absent, decided, resembling* ....
   Consistency, *slimy, butyrous, viscid, membranous, coriaceous, brittle.*
   Medium, *grayed, browned, reddened, blued, greened.*
3. Loeffler's Blood Serum.
   Stroke *invisible, scanty, moderate, abundant.*
   Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
   Elevation of growth, *flat, effuse, raised, convex.*
   Luster, *glistening, dull, cretaceous.*
   Topography, *smooth, contoured, rugose, verrucose.*
   Chromogenesis (8) ....
   Medium, *grayed, reddened, blued, greened.*
   Liquefaction begins in ....d. complete in ....d.
4. Agar Stab.
   Growth *uniform, best at top, best at bottom; surface growth, scanty; abundant; restricted, wide-spread.*
   Line of puncture, *filiform, beaded, papillate, villous, plumose, arborescent; liquefaction.*
5. Gelatin Stab.
   Growth *uniform, best at top, best at bottom.*
   Line of puncture, *filiform, beaded, papillate, villous, plumose, arborescent.*
   Liquefaction, *crateriform, napiform, infundibuliform, saccate, stratiform;* begins in ....d
   complete in ....d.
   Medium, *fluorescent, browned* ....
6. Nutrient Broth.
   Surface growth, *ring, pellicle, flocculent, membranous, none.*
   Clouding, *slight, moderate, strong; transient, persistent; none, fluid turbid.*
   Odor, *absent, decided, resembling* ....
   Sediment, *compact, flocculent, granular, flaky, viscid on agitation, abundant, scant.*
7. Milk.
   Clearing without coagulation.
   Coagulation, *prompt, delayed, absent.*
   Extrusion of whey begins in ....days.
   Coagulum, *slowly peptonized, rapidly peptonized.*
   Peptonization begins on ....d, complete on ....d.
   Reaction, 1d......, 2d......, 4d......, 10d...... 20d....
   Consistency, *slimy, viscid, unchanged.*
   Medium *browned, reddened, blued, greened.*
   Lab ferment, *present, absent.*
8. Litmus Milk.
   Acid, alkaline, acid then alkaline, no change.
   Prompt reduction, no reduction, partial slow reduction.
9. Gelatin Colonies.
   Growth, *slow, rapid.*
   Form, *punctiform, round, irregular, ameboid, mycelioid, filamentous, rhizoid.*
   Elevation, *flat, effuse, raised, convex, pulvinate, crateriform, (liquefying).*
   Edge, *entire, undulate, lobate, erose, lacerate, fimbriate, filamentous, floccose, curled.*
   Liquefaction, *cup, saucer, spreading.*
10. Agar Colonies.
    Growth, *slow, rapid, temperature* ....
    Form, *punctiform, round, irregular, ameboid, mycelioid, filamentous, rhizoid.*
    Surface *smooth, rough, concentrically ringed, radiate, striate.*
    Elevation, *flat, effuse, raised, convex, pulvinate, umbonate.*
    Edge, *entire, undulate, lobate, erose, lacerate, fimbriate, floccose, curled.*
    Internal structure, *amorphous, finely-, coarsely-granular, grumose, filamentous, floccose, curled,*
11. Starch Jelly.
    Growth, *scanty, copious.*
    Diastasic action, *absent, feeble, profound.*
    Medium stained ....
12. Silicate Jelly (Fermi's Solution).
    Growth, *copious, scanty, absent.*
    Medium stained ....
13. Cohn's solution.
    Growth, *copious, scanty, absent.*
    Medium, *fluorescent, non-fluorescent.*
14. Uschinsky's Solution.
    Growth, *copious, scanty, absent.*
    Fluid, *viscid, not viscid.*
15. Sodium Chloride in Bouillon.
    Per cent inhibiting growth ....
16. Growth in Bouillon over Chloroform, *unrestrained, feeble, absent.*
17. Nitrogen. Obtained from *peptone, asparagin, glycocoll, urea, ammonia salts, nitrogen.*
18. Best media for long-continued growth ....
    ....
19. Quick tests for differential purposes ....
    ....
    ....

III. PHYSICAL AND BIOCHEMICAL FEATURES.

Fermentation-tubes containing peptone-water or sugar-free bouillon and	Dextrose	Saccharose	Lactose	Maltose	Glycerin	Mannit				
Gas production, in per cent										
$\left(\frac{H}{CO_2}\right)$										
Growth in closed arm										
Amount of acid produced 1 d.										
Amount of acid produced 2 d.										
Amount of acid produced 4 d.										

2. Ammonia production, *feeble, moderate, strong, absent, masked by acids.*
3. Nitrate in nitrate broth,
   Reduced, not reduced.
   Presence of nitrites ....ammonia ....
   Presence of nitrates ....free nitrogen ....
4. Indol production, *feeble, moderate, strong.*
5. Toleration of Acids, *great, medium, slight.*
   Acids tested ....
6. Toleration of NaOH, *great, medium, slight.*
7. Optimum reaction for growth in bouillon, stated in terms of Fuller's scale ....
8. Vitality on culture media, *brief, moderate, long.*
9. Temperature relations:
   Thermal death-point (10 minutes, exposure in nutrient broth when this is adapted to growth of organism) ....C.
   Optimum temperature for growth ....C.: or best growth at 15° C., 20° C., 25° C., 30° C., 37° C., 40° C., 50° C., 60° C.
   Maximum temperature for growth ....C.
   Minimum temperature for growth ....C.
10. Killed readily by drying, resistant to drying.
11. Per cent killed by freezing (salt and crushed ice or liquid air) ....
12. Sunlight, exposure on ice in thinly sown agar plates; one-half plate covered (time 15 minutes), *sensitive, not sensitive.*
    Per cent killed ....
13. Acids produced ....
14. Alkalies produced ....
15. Alcohols.....
16. Ferments, *pepsin, trypsin, diastase, invertase, pectase, cytase, tyrosinase, oxidase, peroxidase, lipase, catalase, glucase, galactase, lab,* etc. ....
    ....
17. Crystals formed ....
18. Effect of germicides:

Substance	Method used.	Minutes	Temperature	Killing quantity	Amt. required to restrain growth

IV. PATHOGENICITY.

1. Pathogenic to Animals.
   Insects, crustaceans, fishes, reptiles, birds, mice, rats, guinea pigs, rabbits, dogs, cats, sheep, goats, cattle, horses, monkeys, man ....
2. Pathogenic to Plants:
   ....
   ....
   ....
3. Toxins, *soluble, endotoxins.*
4. Non-toxin forming.
5. Immunity bactericidal.
6. Immunity non-bactericidal.
7. Loss of virulence on culture media: *prompt, gradual, not observed* in ....months.

BRIEF CHARACTERIZATION

Mark + or O, and when two terms occur on a line erase the one which does not apply unless both apply.

MORPHOLOGY. (2)		Diameter over 1μ	
		Chains, filaments	
		Endospores	
		Capsules	
		Zooglœa, Pseudozooglœa	
		Motile	
		Involution forms	
		Gram's Stain	
CULTURAL FEATURES. (3)	Broth	Cloudy, turbid	
		Ring	
		Pellicle	
		Sediment	
	Agar	Shining	
		Dull	
		Wrinkled	
		Chromogenic	
	Gel. Plate	Round	
		Proteus-like	
		Rhizoid	
		Filamentous	
		Curled	
	Stab Gel.	Surface-growth	
		Needle-growth	
	Potato	Moderate, absent	
		Abundant	
		Discolored	
		Starch destroyed	
		Grows at 37° C.	
		Grows in Cohn's Sol.	
		Grows in Uschinsky's Sol.	
BIOCHEMICAL FEATURES.	Liquefaction	Gelatin (4)	
		Blood-serum	
		Casein	
		Agar, mannan	
	Milk	Acid curd	
		Rennet curd	
		Casein peptonized	
		Indol (5)	
		Hydrogen sulphide	
		Ammonia (5)	
		Nitrates reduced (5)	
		Fluorescent	
		Luminous	
DISTRIBUTION.		Animal pathogen, epizoon	
		Plant pathogen, epiphyte	
		Soil	
		Milk	
		Fresh water	
		Salt water	
		Sewage	
		Iron bacterium	
		Sulphur bacterium	

Age of gelatin colony	......days	......days	......days
Size of colony			
Surface elevation			

Gelatin colony Titre.............. Incubated at ....° C.	

Litmus milk	Acid	
	Gas	
	Acid curd	
	Rennet curd	
	Reduction	
	Alkali	
	Peptonization	

Fermentations

% Of gas in	Control	Dextrose	Lactose	Saccharose	Glycerin
24 hours					
48 hours					
3 days					
7 days					
Total gas production					
Ratio of $CO_2:H_2$ and other gases					
Acid					
Growth in closed arm					

Chromogenesis on	Nutrient broth	
	Nutrient gelatin	
	Nutrient agar	
	Potato	
Production of	NH_3 from peptone	
	H_2S from peptone	
	Indol from peptone	
	Nitrites from peptone	
Reduction of nitrates to	NH_3	
	Nitrites	

Remarks:

EXERCISE 46. TO DEMONSTRATE THE EFFICIENCY OF INTERMITTENT HEATING AS A METHOD OF STERILIZING MEDIA. ALSO TO COMPARE THE EFFICIENCY OF CONTINUOUS AND INTERMITTENT HEATING

Apparatus. 400 c.c. fresh skim milk; forty sterile test tubes; 2% azolitmin solution.

Method. **1.** Prepare litmus milk according to directions on p. 24.

2. Fill the tubes, using approximately 8 c.c. per tube; mark tubes corresponding to time of heating in sets and each tube in the set, e.g., Set 1 Tube *a*, etc., so as to identify them.

3. Set five away without heating.

Heat five for fifteen minutes on the first day.

Heat five for one hour on the first day.

Heat five for fifteen minutes on two successive days.

Heat five for fifteen minutes on three successive days.

Heat five for fifteen minutes on four successive days.

Heat ten for fifteen minutes on five successive days.

4. Keep all tubes at room temperature. Examine every two or three days and describe the macroscopical changes of each set, as described under the discussion on litmus milk (pp. 24–25.)

Why do not all the tubes of a set change alike? Why do not all sets present the same appearance?

Save all tubes that do not show macroscopical changes. These are probably sterile.

5. Tabulate your results after ten days to two weeks, recording the number and per cent of each lot that shows macroscopical changes.

6. Is milk difficult to sterilize? Why? What other media present the same problem of sterilization as milk? Why?

Would any other method for the sterilization of milk be preferable to the ones you used? Give reasons for your answer.

7. State your results in detail and point out any conclusions that may be drawn and any practical applications that may be made.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 218–221, 378–386, 393–396, 438–449.

Conn, H. W.: Bacteria, Yeasts and Molds, pp. 191–193.

Besson, A.: Practical Bacteriology, Microbiology and Serum Therapy, pp. 35–36.

EXERCISE 47. TO COMPARE MORPHOLOGICALLY PROTOZOA WITH BACTERIA

Apparatus. Deep culture dish; concave slide; clean cover-glasses; cover-glass forceps; platinum loop; tube of sterile broth; tube of sterile Chinese ink.

Cultures. Rich soil or slimy leaves from a pond.

Method. 1. Place the soil or leaves in the deep culture dish.

2. Fill the dish two-thirds full with tap water and add the contents of a tube of broth.

3. Keep the dish at room temperature for twenty-four to forty-eight hours.

4. At the end of the incubation period, make a hanging drop from the supernatant liquid. Before inverting the drop on the slide, add to it that amount of Chinese ink that adheres to the end of a platinum needle.

By the use of this ink, organisms are brought out by contrast, showing white on a dark field. The organisms are not killed or injured by the ink.

5. Observe and measure any protozoa, using the lowest power objective with the step micrometer. Record the size in micra.

6. Roughly sketch the different species observed, giving comparative measurements.

7. Using the highest power dry objective, observe bacteria, noting morphology and size.

8. Draw lines to represent the ratio between the size of the predominant types of each.

9. Are the protozoa present visible to the naked eye?

10. How do the protozoa and bacteria in the drop compare in numbers? Do these organisms have any relation to each other? If so, explain.

11. Of what importance are protozoa? Name several well-known protozoa.

12. State your results in detail and point out any conclusions that may be drawn and any practical applications that may be made.

REFERENCE

Marshall: Microbiology, Second Edition, pp. 12–14, 120–139, 142–145, 264, 305–308, and 669.

EXERCISE 48. TO STUDY THE NATURAL DECOMPOSITION OF MILK

Apparatus. 500 c.c. sterile Erlenmeyer flask; two 5 c.c. sterile pipettes; ten 1 c.c. sterile pipettes; four 10 c.c. sterile pipettes; six 200 c.c. sterile Erlenmeyer flasks; fifteen sterile Petri dishes; physiological salt solution.

Cultures. Fresh skim milk.

Method. 1. Prepare "dilution flasks" as given in Exercise 13, p. 50, making two 90 c.c. and four 99 c.c. flasks. Sterilize by heating one hour in flowing steam or five minutes in the autoclav at 120° C. (15 lbs. pressure). *Dilution flasks and all glassware must be sterile before the experiment proper can be started.*

2. Place 200 c.c. of the fresh skim milk in the sterile

500 c.c. flask and use this sample for the entire experiment.

3. Plate the milk immediately on nutrient agar, using dilutions according to the age of the milk, as follows. (See Exercise 13, p. 50, for method of using dilution flasks.)

Age.	Dilutions.
Fresh milk.........	1 : 1,000, 1 : 10,000 and 1 : 100,000
One day old........	1 : 10,000, 1 : 100,000 and 1 : 1 M *
Four days old.......	1 : 10 M, 1 : 100 M and 1 : 1,000 M
Eight days old......	1 : 10 M, 1 : 100 M and 1 : 1,000 M
Ten days old.......	1 : 1 M, 1 : 10 M and 1 : 100 M

Keep the plates at room temperature.

Sterile pipettes are to be used *always* in making dilutions, plating and titrating.

After the milk curdles it is advised to make the first dilution 1 : 10 to give a more uniform sample, from which further dilutions are made. Use a 10 c.c. pipette having a large opening in the delivery end to prevent clogging.

4. Titrate the milk sample every day. After the milk curdles, shake well before titrating and choose a 5 c.c. pipette having a large aperture for delivery for obtaining the sample for titration.

5. Record the reaction in per cent normal.

After using pipettes, dilution flasks, etc., clean, refill and sterilize them at once for future use.

6. Note the *macroscopical changes* in the milk sample (due to microbial growth), e.g., kind and consistency of curd, extrusion of whey, gas formation, peptonization; also note odor from time to time.

7. Note the *macroscopical evidences of microbial growth* such as molds, etc., and the time of appearance. Identify the group to which these organisms belong, giving genus and species if possible.

* M = Million.

8. Determine the changes in the numbers of microorganisms by counting the colonies of the different sets of plates after they have developed seven days at room temperature (see p. 54, Exercise 14, for method).

9. Estimate the number of colonies of each type (see Exercise 14, p. 54.)

10. Record your results, noting the *date* on which the plates were made, the *age* of the milk, the *dilution, number* of colonies *on the plate* and the *average number* of organisms *per cubic centimeter.*

11. Examine in hanging drop and note the morphology of the microorganisms producing the most predominant types of colonies on each set of plates. Indicate after the drawing, the comparative numbers on each set of plates by the signs −, +−, +, ++, etc., to indicate absence, presence of few, or many of the type.

12. Note whether molds or yeasts are present on any set of plates. Should either be found on fresh milk plates? Why? What types of microorganisms would you expect on fresh milk plates?

13. Prepare your data according to the following diagram:

Date	Age	Acidity	Dilution	Count per cc.	Organisms	
					Types	Relative Nos.
Feb. 2	Fresh	+15°	1–1,000	278,900	Acid	+
	(26 hrs. old)		1–10,000	325,500	Yellow	+−
			1–100,000	300,000		

Average count per cc.............. 301,470

14. Plot the curve showing the change in acidity and one illustrating the count per cubic centimeter on the same paper, using different colored inks or different types of lines. Use days for abscissæ, acidity and count for ordinates. Start at the same origin.

15. Is there any relation between the change in acidity and the change in flora?

Should the acidity and count curves run parallel? If they do not, give a reason why.

How could the bacterial count be made to increase after it goes down to a constant number?

16. What biochemical changes have occurred in the decomposition?

17. Compare the flora of fresh milk, 7.0% normal acid milk and ten-day milk, both microscopically and from the plates. Explain.

18. State your results in detail and give any conclusions to be drawn and any practical applications that may be made.

REFERENCES

CONN: Practical Dairy Bacteriology, pp. 21–57, 81–85.
MARSHALL: Microbiology, Second Edition, pp. 378–396.

EXERCISE 49. TO ISOLATE SPORE-FORMING BACTERIA AND TO STUDY SPORE FORMATION

Apparatus. Two tubes of sterile broth; small piece of hay; three sterile Petri dishes; clean test tube; three tubes sterile agar for plates; three sterile agar slants; carbol-fuchsin; acetic acid alcohol; aqueous-alcoholic methylen blue; platinum needle and loop; ordinary forceps.

Culture. Hay.

Method. **1.** Place a piece of hay in the clean test tube, plug the tube, and sterilize in the hot-air oven.

2. Using sterile forceps, place the sterile hay "aseptically" in one tube of broth and an unsterilized piece in the other.

3. Incubate both at room temperature for forty-eight hours. Do both tubes show growth?

4. Heat in a water-bath at 80° C. for ten minutes the

tube which shows marked growth. What does this accomplish?

5. Make three loop-dilution plates from the heated broth tube.

6. Place the plates at room temperature and examine them daily for colony development.

7. Make pure cultures on agar slants from three different well-isolated colonies of the predominant types and incubate at room temperature.

8. Examine these in thirty-six to forty-eight hours in a hanging drop for morphology and spore formation.

9. Make a spore stain as soon as spores are found. Where are the spores located in the bacterial cell?

10. Have you studied any pure culture of bacteria which is similar to the types you have isolated? What organism is commonly found in hay? In what form does it exist on the hay? What do you know of the habitat of this organism and related forms? Of the pathogenicity?

11. State the results obtained in detail; draw the conclusions which follow and point out any possible practical applications.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 5, 82–84, 219–221, 251, and 310.

JORDAN: General Bacteriology, Fifth Edition, pp. 245–246.

EYRE: Bacteriological Technic, Second Edition, p. 140.

EXERCISE 50. TO DEMONSTRATE THE EFFICIENCY OF FILTRATION AS A MEANS OF REMOVING MICROORGANISMS FROM LIQUIDS

Apparatus. Six small funnels; two small filter papers; two small pieces of absorbent cotton; two small pieces of clean hospital gauze; eight tubes sterile agar; eight sterile Petri dishes; ten sterile 1 c.c. pipettes; sterile 10 c.c.

pipette; three dilution flasks; six sterile test tubes; tube of sterile broth.

Culture. *B. coli.*

Method. **1.** Inoculate broth with *B. coli* and incubate for twenty-four hours at 21° C.

2. Sterlize filter paper in each of the two small funnels, a small piece of absorbent cotton in each of two more; fold two pieces of clean gauze several thicknesses and sterilize in the remaining two funnels. Wrap all in paper and sterilize in the hot-air oven.

3. Shake the broth culture of *B. coli* and plate, using dilutions 1 : 1,000 and 1 : 10,000.

4. Filter each dilution (1 : 1,000 and 1 : 10,000) through each of the different substances, catching the filtrate in sterile test tubes.

5. Plate 1 c.c. from each filtrate immediately and incubate the plates at 21° C.

6. At the end of five days, count the plates.

7. Which method of filtration is most efficient? Why? What factors could greatly influence the numbers of microorganisms developing on the plates after filtration?

8. What methods are most efficient in removing microorganisms from liquids? Why?

9. Suggest some natural methods of filtering microorganisms.

10. Give in detail the results obtained, state any conclusions that may be drawn and point out any practical applications.

REFERENCES

Eyre: Bacteriological Technic, 2d Ed., pp. 42–48.
Marshall: Microbiology, Second Edition, pp. 116–119, 387–388.

EXERCISE 51. TO DEMONSTRATE PRESENCE OF MICROORGANISMS IN AIR, ON DESK, FLOOR, ETC.

Apparatus. Seven sterile Petri dishes; seven tubes of sterile agar.

I. *Air.* **Method.** **1.** Pour seven plates with uninoculated sterile agar and set on a level surface until solid.

2. Expose one plate for one minute to (*a*) laboratory air; (*b*) air of campus; (*c*) air of your room while sweeping or dusting.

II. *Floor.* **1.** Bend the straight platinum needle till it forms a right angle.

2. Sterilize it in the flame.

3. Moisten the needle with sterile water.

4. Rub it along the floor, and then,

5. Draw it lightly across the surface of the agar in the fourth Petri dish.

III. *Desk.* **1.** Sterilize the needle and repeat operation (II, **5**) obtaining the inoculum from the surface of a desk which has *not* just previously been washed with 1 : 1,000 mercuric chloride.

2. Then wash the surface of the desk well with this solution and when the desk top is dry, repeat the operation, using the sixth plate. Leave the seventh plate uninoculated for control.

3. Mark all plates with the date on which they were exposed or inoculated and place them at a constant temperature.

4. Watch any developments from day to day. What organisms predominate on the plates? Why?

5. Examine different colonies in a hanging drop. What types of bacteria are found?

6. Upon what does the species and number of microorganisms depend? What becomes of them when air currents are present? When the floor is swept in the ordinary

way? Mopped? When the desk is washed with water? With mercuric chloride?

7. Are these types deleterious to health? Why should and how may they be avoided in the laboratory? Outside of the laboratory?

8. State your results for I, II and III in detail, draw any conclusion possible and point out any practical operations.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 247 and 253.
BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 862–863.
CONN: Bacteria, Yeasts and Molds, pp. 114–123.
CONN: Practical Dairy Bacteriology, pp. 65–67.
TYNDALL: Floating Matter of the Air.

EXERCISE 52. QUALITATIVE STUDY OF THE MICROFLORA OF THE SKIN AND HAIR IN HEALTH AND IN DISEASE

Apparatus. Ordinary forceps; two sterile, small white enamel basins (*steam-sterilized*); three sterile 1 c.c. pipettes; six sterile Petri dishes; six tubes of sterile agar; one liter flask containing about 700 c.c. of sterile water or salt solution; sterile cloth ($\frac{1}{2}$ yd. hospital gauze wrapped in paper and sterilized at 180° C.); soap; clean slides and cover-glasses.

Method. I. *Skin.* (*a*) *Normal.* **1.** Place about half the sterile water in a sterile basin.

2. Wash the hands thoroughly in the sterile water.

3. Plate 1 c.c. of this water immediately in ordinary agar.

4. Then wash the hands well with soap and tap water, rinse with tap water till free of soap and dry the hands on the sterile cloth.

5. Place the remaining sterile water in the second sterile basin and wash the hands again.

6. Plate 1 c.c. of this water.

7. Incubate both plates (inverted) at 37° C.

8. How long before starting this experiment did you wash your hands? How might this influence your results?

9. What types of microorganisms would you expect to find on the skin? Why?

(*b*) *Diseased.* **1.** With a sterile needle obtain a small amount of purulent material from a pustule, boil, or abscess, etc.

2. Make three loop-dilution plates in agar. Incubate at 37° C.

3. Examine some of this material microscopically by preparing a stained smear.

4. Draw and describe the latter. Do you find the same organisms on the plates as on the slide?

5. *Isolate the most predominant organisms on the plates and identify them.*

6. What is the source of all these microorganisms? What becomes of them when we wash our hands and wipe them in the ordinary way? Are they detrimental to health?

7. What is pus? Of what does it consist? What care should be taken with discharges from suppurating sores?

II. *Hair.* (*a*) *Normal.* **1.** Using flame-sterilized forceps (ordinary type), obtain several hairs and place them in a sterile Petri dish.

2. Using a sterile pipette, add 1 c.c. of sterile water or salt solution to the Petri dish and stir the hairs about in it with the pipette or sterile loop in order to dislodge the organisms adhering to them.

3. Pour into the plate a tube of melted agar (at 40° to 45° C.), and when hard, incubate at 37° C.

4. After twenty-four to forty-eight hours, examine predominating colonies in a hanging drop.

(*b*) *Diseased.* **1.** With sterile forceps obtain a few hairs

from the growing edge of the infected portion of the skin affected with ringworm or barber's itch. These hairs will come out easily in comparison with healthy hairs.

2. Mount and examine for the fungus, *Trichophyton tonsurans*. (See illustration on p. 726 in Marshall's Microbiology.) Draw.

3. Continuous application of a glycerinated solution of 1 : 500 $HgCl_2$ (glycerin 1 part, $HgCl_2$ 1 : 500, 9 parts) will kill this fungus.

State in detail your results for I and II, draw any conclusion permissible and point out any practical application.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 662–664, 666, 668, 669, 688, 689, 726, 727, 741–743.

BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 679–688.

EXERCISE 53. QUALITATIVE STUDY OF THE MICROFLORA OF THE MUCOUS MEMBRANE (MOUTH AND THROAT OR NOSE)

Apparatus. Absorbent cotton, small piece; wire rod about 15 cms. long; clean slides and cover-glasses; Petri dish, sterile; tube of sterile agar; tube of sterile broth; aqueous-alcoholic fuchsin.

Method. I. *For Teeth.* **1.** Place a small drop of distilled water on a clean cover-glass or slide.

2. Introduce some material obtained by scraping along the base of and between the teeth with a sterile platinum needle.

3. Allow to dry, fix and stain with aqueous-alcoholic fuchsin.

4. Examine microscopically with the oil immersion lens.

5. Draw all forms seen. Would all of these forms grow on an agar plate? Give reasons for your answer.

II. *For Throat or Nose.* **1.** Prepare a swab by winding

a small piece of absorbent cotton snugly about one end of the wire rod.

2. Place in a test tube, swab end down, and prepare for sterilization as with pipettes.

3. Dry-sterilize.

4. Pour an agar plate and allow it to harden.

5. Moisten the sterile swab in sterile broth, using aseptic precautions, and then swab the throat or nose.

6. Lightly brush the inoculated swab over the surface of the agar plate and place the plate inverted, at 37°, to develop.

7. Using the same swab, make a smear on a clean glass slide, dry, fix, stain and examine as with the preparation from the teeth.

8. Return the swab to the tube of broth, incubate at 37° for twenty-four hours and examine the growth in a hanging drop.

9. Draw and describe the predominating organisms.

10. Find a streptococcus, if possible, on the agar plate from the swab.

11. Make a stained slide and have the instructor inspect the same, when you think that you have been successful.

12. How does the microflora of the mucous membrane differ from that of the outer skin?

CAUTION. Aseptic precaution must be taken in all instances, as some of the microorganisms may be pathogenic!

State in detail your results from I and II, draw any conclusions possible and point out any practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 496–500, 662–664, 665, 668, 670–672, 689–692, and 734.

MacNeal: Pathogenic Microorganisms, pp. 261, 264 and 366.

Besson: Practical Bacteriology, Microbiology and Serum Therapy, pp. 81, 191, 197, 270, 592–610, 617–626.

PART II

PHYSIOLOGY OF MICROORGANISMS

EXERCISE 1. TO DEMONSTRATE THE SMALL AMOUNT OF FOOD NEEDED BY BACTERIA

Apparatus. Distilled water; sterile Petri dishes; sterile 1 c.c. pipettes; sterile 10 c.c. pipettes; eight sterile 200 c.c. flasks; nineteen tubes of sterile plain agar.

Cultures. *B. coli.*

Method. **1.** Place 150 c.c. of distilled water in each of two sterile flasks.

2. Sterilize one flask (flask *B*) in the autoclav for ten minutes at 15 lbs. pressure (120° C.).

3. Plate 1 c.c. from the remaining flask (flask *A*), immediately on agar.

4. As soon as flask *B* is cold, plate 1 c.c.

5. Then inoculate the water in flask *B* with *B. coli,* using the *straight needle and transferring a very small amount,* and plate 1 c.c.

6. Place flasks *A* and *B* and the plates made from the flasks at room temperature.

7. Prepare four 90 c.c. and two 99 c.c. dilution flasks and sterilize.

8. At the end of two days and at the end of five days, plate from flasks *A* and *B*, using 1 c.c. direct and dilutions 1 : 10, 1 : 100 and 1 : 1000. Incubate the plates at room temperature.

9. Count each set of plates at the end of five days' incubation.

10. Compute the weight of the bacteria in the flask of distilled water at its highest count.

What is the smallest amount that may be weighed on the ordinary analytical balances? Conclusions?

11. Plot curves to show whether bacteria are decreasing or increasing. Offer a logical explanation for the direction the curve takes in each instance.

12. Note the conditions under which distilled water is obtained and dispensed in the laboratory. Why is the distilled water not sterile?

13. By what process of distillation may distilled water be obtained free from microorganisms? What several factors outside of errors in technic may have influenced your results?

14. What would be the comparative influence of a large and a small inoculation upon the number of *B. coli* surviving the 5 days sojourn in the distilled water?

15. State your results in detail, draw any possible conclusions and point out any practical applications.

REFERENCES

Marshall, C. E.: Microbiology, Second Edition, pp. 149–151.
Fischer, Alfred: Structure and Functions of Bacteria, pp. 52–54.
Prescott and Winslow: Elements of Water Bacteriology, 3d Ed. pp. 151–153.

SOME PHYSIOLOGICAL CLASSIFICATIONS OF BACTERIA

Bacteria are often classified, in general terms, **according to their functions,** into:

Saprogenic, or putrefactive bacteria;
Zymogenic, or fermentative bacteria;
Pathogenic, or disease-producing bacteria.

According to their food requirements, into:

Prototrophic, requiring no organic food (e.g., nitrifying bacteria);

Metatrophic, requiring organic food (e.g., zymogenic bacteria, saprophytes and facultative parasites);
Paratrophic, requiring living food (e.g., obligate parasites); (A. Fischer).

According to special food preferred, into:
Acidophile: acid loving;
Halophile: salt loving;
Saccharophile: sugar loving;
Saprophile: loving dead organic matter;
Coprophile: loving barnyard manure.

According to their oxygen requirements, into:
Aerobic: requiring atmospheric oxygen for growth;
Anaerobic: requiring the absence of atmospheric oxygen;
Partial anaerobic: requiring an intermediate oxygen tolerance.

According to the necessity of one kind of food or environment, into:
Obligate: indicating absolute requirements, e.g., obligate parasite, obligate anaerobe;
Facultative: indicating a variability in requirements; the word following indicates the condition under which the organism may live but does not prefer for growth, e.g., *B. coli* is a facultative anaerobe.

According to their metabolic products, into:
Chromogenic, or pigment-producing bacteria;
Photogenic, or light-producing bacteria;
Aerogenic, or gas-producing bacteria;
Thermogenic, or heat-producing bacteria.

Chromogenic bacteria are classified in accordance with the nature and location of the coloring matter which they elaborate, as

Chromophorus bacteria, the pigment being stored in the cell protoplasm of the organism analogous to the chlorophyll of higher plants, e.g., green bacteria and red sulphur bacteria, purple bacteria.

Chromoparous bacteria, true pigment formers. The pig-

ment is set free as a useless excretion, may be excreted as a colored body or as a colorless substance which becomes oxidized upon exposure to the air. Individual cells are colorless and may cease to form pigment, e.g., *B. prodigiosus, B. ruber, B. indicus.*

Parachrome bacteria. The pigment is an excretory product but is retained within the cell, e.g., *B. violaceus.* (Beijerinck.)

According to their temperature relations, into:

Pecilothermic (*poikilothermic*) bacteria: adaptability to temperature of environment;

Stenothermic bacteria: a very narrow temperature range (strict parasites);

Eurythermic bacteria: a very wide temperature range (metatrophic bacteria), often 30° between maximum and minimum temperatures.

According to their optimum temperature, into:

	Min.	Opt.	Max.
Cryophilic (*psychrophilic*, term used chiefly for water organisms) bacteria..............	0° C.	15° C.	30° C.
Mesophilic bacteria (includes pathogenic bacteria)......	15° C.	37° C.	45° C.
Thermophilic bacteria........	45° C.	55° C.	70° C.

ANAEROBIC CULTURE METHODS

The cultivation of strict anaerobes is accompanied by certain technical difficulties arising from the necessity of removing all traces of oxygen from the medium and from the atmosphere to which this medium is exposed. It is, therefore, necessary to employ special apparatus or special methods for their cultivation.

The recent investigations of Tarrozzi, which confirm the work of Theo. Smith, seem to show that oxygen does not exert any direct harmful effect on anaerobic organisms, but that the presence of free oxygen prevents the medium furnish-

ing the nutritive substances necessary for anaerobic life. Anaerobic organisms can, in fact, as Theo. Smith has shown, be grown in the presence of the oxygen of the atmosphere by simply adding pieces of animal tissue or some reducing agent to the culture medium.

Several principles are employed as a basis for the different methods of anaerobic cultivations, as follows:

I. *Exclusion of air* from the cultivation.

II. *Exhaustion of air* from:

1. The medium by boiling. This should always immediately precede the inoculation of the medium for anaerobic cultivations.

2. The vessel containing the medium by means of an air pump, i.e., cultivation *in vacuo*.

III. *Absorption of oxygen* from the air in contact with the cultivation, i.e., cultivation in an atmosphere of nitrogen, by means of:

1. Chemical action upon a readily oxidizable substance in a sealed vessel containing the cultures, e.g., sodium hydroxide upon pyrogallic acid.

2. Burning a filter paper saturated with alcohol in a sealed vessel. (Moore.) If the paper is well saturated no deleterious products of combustion are formed which would inhibit growth.

3. Adding to the medium some easily oxidizable substance as dextrose (2%), sodium formate (0.5%), sodium sulphindigotate (0.1%) or fragments of sterile tissue to absorb all the available oxygen held in solution by the medium.

The chemicals are generally employed in the case of deep stab cultures, the fragments of sterile tissue in broth cultures (Theo. Smith's method). The tissue must be freshly removed from an animal (rabbit, mouse, guinea pig, etc.) and only pieces of liver, spleen, kidney or lymphatic glands may be used with success; blood, milk, or the connective tissues are useless for the purpose. Vegetable tissue (potato,

elder pith, mushrooms, etc.) have been used similarly with success (Wrzosek, Ori and others). Spongy platinum has also been used similarly with satisfactory results.

The vitality of anaerobic organisms is exhausted much more quickly on media prepared on these principles than on media under anaerobic conditions (Jungano and Distaso).

Perhaps if these methods were used in conjunction with anaerobic methods the vitality of the anaerobes would not be impaired.

4. Growing the anaerobe in the presence of a vigorous aerobe by the use of special methods or apparatus.

IV. *Displacement of air* by an indifferent gas such as hydrogen, carbon dioxid, etc.

V. *A combination of two or more of the above methods.*

The following methods are those best adapted for class use and can be utilized in a regular exercise as desired:

I. EXCLUSION OF AIR

Hesse's Method. This method may be used either with a pure culture or for determining the presence of anaerobes in any substance.

Apparatus. Tubes of agar or gelatin for stab cultures; sterilized oil (olive oil, vaselin or paraffin oil); sterile 1 c.c. pipette.

Culture. Pure culture of an anaerobe.

Method. **1.** Make a stab culture of the anaerobe, using a tube containing a deep column of the medium, and thrusting the inoculating needle to the bottom of the tube. The stab culture and a test tube shake culture also may be treated as follows:

2. With the sterile pipette place a layer of sterile oil,* 1 to 2 cm. deep, upon the surface of the medium.

3. Incubate at the optimum temperature.

* Sterile melted agar or gelatin may be substituted for the sterile oil.

II. EXHAUSTION OF AIR

A. By Boiling. It is well to expel all the air from a medium to be used for isolating or growing anaerobes by boiling twenty to thirty minutes, and cooling rapidly just previous to inoculating, and placing under anaerobic conditions.

B. Cultivation in Vacuo. This requires special apparatus for obtaining a vacuum and for cultivation in some cases.

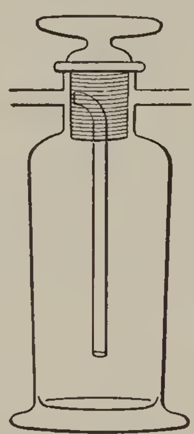


FIG. 39*a*.—Novy Jar for Tube Cultures.

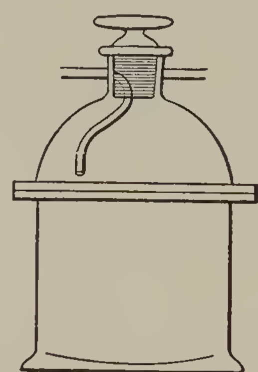


FIG. 39*b*.—Novy Jar for Plate Cultures.

Apparatus. *Special tubes:*

1. Vacuum tubes (Fig. 129, p. 238, Eyre's Bacteriological Technic).

2. Pasteur, Joubert and Chamberland's tube (Fig. 80, p. 93, Besson's Practical Bacteriology, Microbiology and Serum Therapy).

3. Pasteur's tube (Fig. 81, Besson, *ibid.*).

4. Lacomme's tube (Fig. 82, Besson, *ibid.*).

5. Roux's tube for stroke cultures (Fig. 91, p. 101, Besson, *ibid.*).

6. Roux's tube for potato cultures (Fig. 92, p. 101, Besson, *ibid.*).

7. Esmarch's tube (Fig. 95, p. 103, Besson, *ibid.*).

8. Vignal's tube (Fig. 96, p. 103, Besson, *ibid.*).

Special flasks:

1. Pasteur's flask (Fig. 79, p. 92, Besson, *ibid.*).
2. Flasks with long necks (Fig. 83, p. 94, Besson, *ibid.*).
3. Bottle (Fig. 84, p. 94, Besson, *ibid.*).
4. Kitasato's dish (Fig. 93, p. 10, Besson, *ibid.*).
5. Bombicci's dish (Fig. 94, p. 102, Besson, *ibid.*).
6. Ruffer's or Woodhead's flask (Fig. 33, p. 41, Eyre, *ibid.*).

Special jars in which test tube or plate cultures may be placed and a vacuum produced.

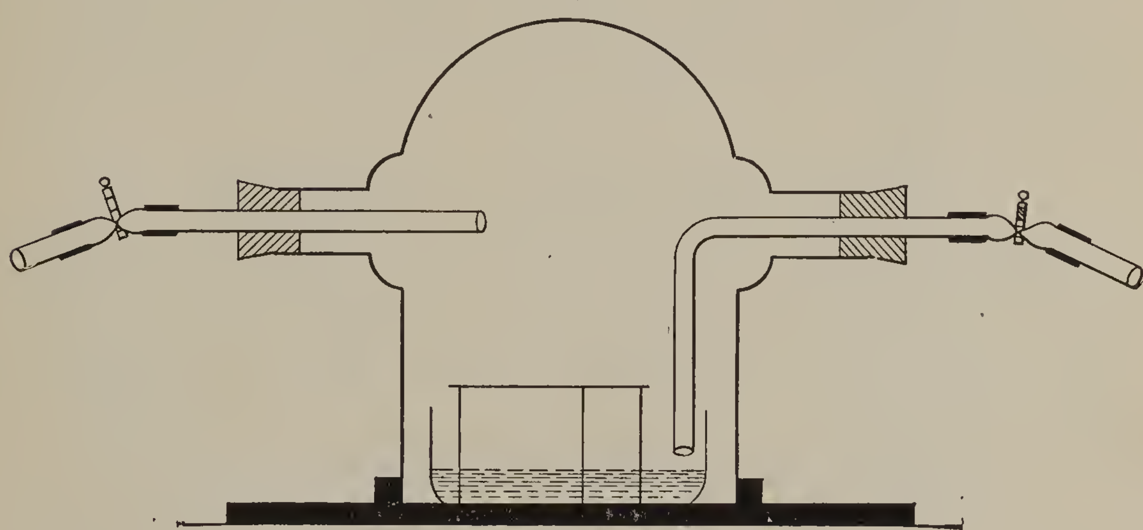


Fig. 40.—Bulloch's Anaerobic Jar.

1. Novy's jar for plates (Fig. 135, p. 245, Eyre, *ibid.*).
2. Novy's jar for tubes (Fig. 136, p. 245, Eyre, *ibid.*).
3. Bullock's anaerobic apparatus (Fig. 137, p. 247, Eyre, *ibid.*).
4. Tretrôp's apparatus (Fig. 97, p. 105, Besson, *ibid.*).
5. Botkin's apparatus (Fig. 134, p. 244, Eyre, *ibid.*).

Apparatus for obtaining a vacuum:

1. Electric pump adaptable to vacuum or pressure.
2. Water vacuum pump.
3. Mercury vacuum pump.

Method. 1. The tube and flask cultivations are prepared by, (*a*) placing the desired medium in the vessel; (*b*) inoculating from the desired source; (*c*) attaching to the vacuum

pump and (*d*) while the pump is running, sealing the tube or flask in the flame, at the constriction provided for the purpose.

2. The special jars have the advantage that tube and plate cultivations may be prepared in the usual way and then placed in the special jar which is then attached to the vacuum pump; when sufficient vacuum has been produced the stopcock is turned between the jar and the pump.

Isolation of anaerobic organisms may be accomplished with much greater facility by the use of these jars.

In practically every instance these same jars may also be employed in the methods given under the absorption of oxygen.

III. ABSORPTION OF OXYGEN

Different methods illustrating this general principle are much used because of its simplicity and general applicability. Any vessel with a tight cover as a Novy jar, an ordinary chemical desiccator, a Mason fruit jar, etc., may be used as a container for the tube or plate culture.

A. Pyrogallic acid method. 1. Dry pyrogallic acid is placed on top of some absorbent cotton in the bottom of the jar or tube.

2. A solution of sodium hydroxide is poured in, but not directly upon it.

3. The cultures are put in place.

4. The jar or tube is immediately sealed and care is taken to mix the chemicals. The organisms thus grow in the presence of the inert gas nitrogen.

The chemicals are used in the proportion of 1 gm. of pyrogallic acid to 10 c.c. of 10% aqueous solution of potassium or sodium hydroxide for each 100 c.c. of air space.

Apparatus. *Tubes* for use in oxygen absorption method.

1. Simple test-tube method.
2. Giltner's H tube.

3. Buchner's tube (Fig. 130, p. 239, Eyre, *ibid.*).
4. Turro's tube (Fig. 86, p. 95, Eyre, *ibid.*).

By the use of these tubes no sealed jar is necessary.

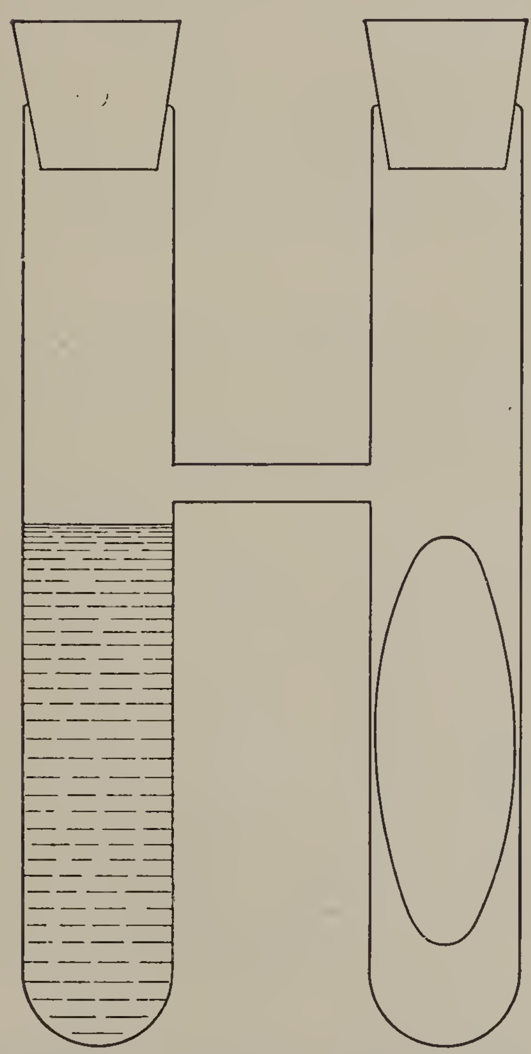


Fig. 41.—Giltner's Tube. (Orig.)

Fig. 42.—Buchner's Tube.

1. The simple test-tube method is advantageous in that it requires no special apparatus. It has disadvantages, however, which will be mentioned later.

Apparatus. Test tube of sterile medium; rubber stopper to fit tube; pyrogallic acid and sodium hydroxide; paraffin.

Method. 1. Inoculate the medium with the material under investigation and replace the plug.

2. Cut off the plug even with the mouth of the tube.

3. Push the plug into the tube, 4 to 5 cm.

4. Place on top of the plug the pyrogallic acid and *only enough* of the alkaline solution to saturate the plug.

5. Insert the rubber stopper and seal with paraffin if necessary. If the cotton is more than saturated, the strong alkaline solution will run through the plug and kill the organisms in the culture.

This preparation is valuable only for noting the presence of anaerobes in any substance or studying the growth of an anaerobe in pure culture, on account of the difficulties of technic.

2. *Giltner's H Tube.* This is simply two test tubes connected near their mouths by a short piece of glass tubing. By this method the tube cultivation may be placed in one test tube, the chemicals in the other and both tubes stoppered. (Fig. 41, p. 159).

The use of this apparatus presents a distinct advantage over any other tube cultivation method, as the culture is readily discernible at all times and may be handled without the disagreeable features of the other methods.

The H tube lends itself also to the method depending upon the absorption of oxygen by an aerobic organism.

3. *Buchner's tube* consists of a stout glass test tube having dimensions of about 23 cm. in length and 4 cm. in diameter, fitted with a rubber stopper.

a. A test-tube culture of the organism or mixed culture to be tested is prepared.

b. A little cotton, the pyrogallic acid, and sodium hydroxide solution are placed in the Buchner tube, the culture *immediately* introduced and the rubber stopper *immediately* fitted tightly in the mouth of the large tube. (Fig. 42, p. 159).

4. *In Turro's tube,* the medium is poured through the

small inner tube, sterilized and inoculated. The pyrogallic acid and sodium hydroxide are then placed in the bulb and the stopper immediately replaced.

This method has advantages over Buchner's in that the oxygen is much more rapidly absorbed and the culture is visible during incubation.

Plates. 1. Ordinary deep culture (Petri) dish.

2. McLeod's plate base (used with the bottom of a deep Petri dish). (Muir and Ritchie, 6th Ed., Fig. 23, p. 65.)

The principle of using these two plates is the same throughout and is illustrated in Exercise 2.

Jars. As has been noted before, the jars designed for obtaining vacuum may be utilized in the pyrogallic acid method and in the method making use of burning alcohol to exhaust the oxygen.

B. Liborius-Veillon Method and Roux's Biological Method depend upon the *abstraction of oxygen from the medium by aerobic organisms.* Liborius makes use of the aerobes already present in the mixed culture, while Roux uses a pure culture of an obligate aerobe. Nowak first grew *Bact. abortus* by this method.

Liborius-Veillon Method. 1. Fill long test tubes (22 cm. × 1.5 cm.) to a depth of 10–15 cm. with glucose agar or gelatin and sterilize (below 120° C.).

2. Place the tubes in a water bath and boil twenty to thirty minutes to liquefy the agar and drive off the air dissolved in the medium; then cool to 40°–45° C. until sown.

3. Make loop dilutions in the melted agar and, as soon as the tubes are sown, cool them rapidly in an upright position.

Aerobic organisms grow in the upper part of the medium which contains a certain amount of air in solution, while the anaerobes multiply in the deeper layer.

Roux's Method. 1. Make a deep agar or gelatin stab or shake culture of the organism or substance to be studied.

2. Pour upon the surface of this medium a layer 1 to 2 cm. deep of a broth culture of a vigorous obligate aerobe as

B. subtilis, or an equal depth of liquefied agar or gelatin and inoculate this when solid with the aerobe.

The growth of the aerobe will use up all the oxygen that reaches it and will not allow any to pass through to the medium below, which will consequently remain in an anaerobic condition.

Giltner's H-tube Method. The use of Giltner's H-tube allows the anaerobe in a certain medium to be grown on one side of the H either as a stab culture or a streak, while the aerobe in the same or a different medium, liquid or solid, may be grown on the other side. Rubber stoppers, fitted to mouths of both tubes, are superimposed on cotton plugs. The aerobe soon exhausts the oxygen from the tube, allowing the anaerobes to develop.

This is the method recommended for determining the presence of and isolating *Bact. abortus* from infected mucous membranes and tissues. This organism when first isolated from tissues is a partial anaerobe, i.e., when an agar shake culture is made in an ordinary test tube the colonies develop in a *zone* about 0.5 cm. in width about 1.5 to 2 cm. below the surface of the agar.

By the use of the H tube, surface colonies of this organism may be readily obtained for study.

Novy Jar Method. This same principle may be applied by the use of separate tube or plate cultivations of anaerobes and aerobes in a Novy jar or similar apparatus; the aerobic organisms should be offered a large surface for growth in each case.

IV. DISPLACEMENT OF AIR BY INDIFFERENT GASES

The special tubes, flasks and jars adapted to cultivation of anaerobes in a vacuum are equally applicable in this method.

The gas generally employed is hydrogen. It is preferable to other gases not only because it is easily prepared, but that it has no injurious effects on the organisms.

A Kipp generator is connected up with three wash bottles, containing:

(*a*) 10% lead acetate solution to remove H_2S;

(*b*) Silver nitrate solution to remove AsH_3;

(*c*) 10% pyrogallic acid solution made alkaline to remove any trace of oxygen, may be used to furnish hydrogen.

Hydrogen is most conveniently obtained by keeping a cylinder of the compressed gas in the laboratory. This gas generally contains about 99.6% hydrogen, the remaining 0.4% is mostly or entirely air, which represents 0.08% oxygen. The gas so kept requires no preliminary washing, but may be passed direct from the cylinder into the jar or flask.

Carbon dioxide is harmful to a large number of organisms, as is also coal gas. Nitrogen is satisfactory, but its method of preparation is so difficult that its use should be abandoned in practical bacteriology unless it can be obtained compressed in cylinders.

EXERCISE 2. THE EFFECT OF ANAEROBIC CONDITIONS UPON MICROORGANISMS FROM MANURE

Apparatus. Modeling clay; tubes of sterile gelatin; three sterile Petri dishes; three sterile deep-culture dishes (use top of Petri dish for cover); sterile 1 c.c. pipettes; sterile dilution flasks; six tubes of sterile agar; pyrogallic acid; 10% solution of sodium hydrate; absorbent cotton.

Culture. Horse manure.

Method. **1.** Plate the manure (1 gm. in 99 c.c. dilution flask) in duplicate in the Petri dishes and in the deep culture dishes, using dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000.

2. As soon as the agar is solid, invert the deep culture dishes containing the dilutions.

3. Place a *small* piece of absorbent cotton in the center of the cover. This must not touch the agar.

4. On the absorbent cotton, place 1 gm. of pyrogallic

acid crystals; then place 10 c.c. of 10% NaOH in the cover of the dish. (The cotton prevents a too rapid reaction between the chemicals.)

5. Seal *at once* by packing the space between the cover and bottom air tight with modeling clay. Then mix the chemicals.

6. Place all six plates at room temperature.

Note. In the reaction which takes place between pyrogallic acid and NaOH, oxygen is used and an anaerobic condition is established within the culture dish (exact reaction not known.)

7. Count the organisms after seven days. Estimate the number of different types of colonies developing under the varying conditions of air supply and note growth. Conclusions?

8. Compare your results with those of others and draw conclusions.

9. Make gelatin stabs of three or four of the predominant types of colonies on the anaerobic plates and cultivate anaerobically by Hesse's method. What types of organisms are these morphologically and culturally?

10. Are any types found on aerobic plates which are lacking on the anaerobic plates and vice versa?

What type of anaerobe is frequently found in horse manure?

11. When do anaerobic conditions exist in milk? In soil? Is this beneficial or otherwise in each case? What relation may there be between age of milk and type of colonies? Can this same relationship apply in the case of soil?

12. What other methods may be used for obtaining anaerobic conditions for microbial growth? Name the obligate anaerobes.

13. How is an organism isolated which is tolerant of an amount of oxygen less than that of the atmosphere, but will not grow under strictly anaerobic conditions?

14. State your results for the experiment in detail and

point out any conclusions that may be drawn. Mention any practical applications to be made.

REFERENCES

Eyre: Bacteriological Technic, Second Edition, pp. 236–247.

Marshall: Microbiology, Second Edition, pp. 153–157, 281, 292–295, 378–386, 512, 784–792.

Besson: Practical Bacteriology, Microbiology and Serum Therapy, pp. 87–105.

Giltner: Suggestions for partial anaerobic cultures. Science, n. s., Vol. XLI, No. 1061, p. 663.

EXERCISE 3. TO DEMONSTRATE THAT ACIDS ARE FORMED FROM CARBOHYDRATES BY BACTERIA

Apparatus. Three tubes of sterile litmus lactose agar; three tubes of sterile dextrose agar containing $CaCO_3$; sterile dilution flask (containing about 150 c.c. sterile salt solution); six sterile Petri dishes; sterile 1 c.c. pipettes.

Culture. Fresh milk culture of *Bact. lactis acidi.*

Method. **1.** Place a *very small* loopful of the *Bact. lactis acidi* culture in the dilution flask. (Transfer from the white portion of the litmus milk culture.) Shake well.

2. Make three plates from each medium, using widely varying amounts, for example, 0.5 c.c., 0.1 c.c. and 1 drop. *Just before plating,* mix the $CaCO_3$ *well* with the agar (avoid air bubbles).

3. Place the plates (inverted) at room temperature.

4. Examine each daily after forty-eight hours. Note how each medium is changed by the growth of the colonies. Explain what has happened.

How is the object of the experiment demonstrated in the case of each medium?

5. Compare the size of colonies on the different media; also on each dilution of one medium; explain. Why are the colonies smaller on the thickly seeded plates?

6. Write the chemical equation with a specific enzyme for each change in the case of each medium.

7. Write the reaction involving the $CaCO_3$.

8. State your results for the experiment in detail, draw any conclusions and point out any practical applications that may be made.

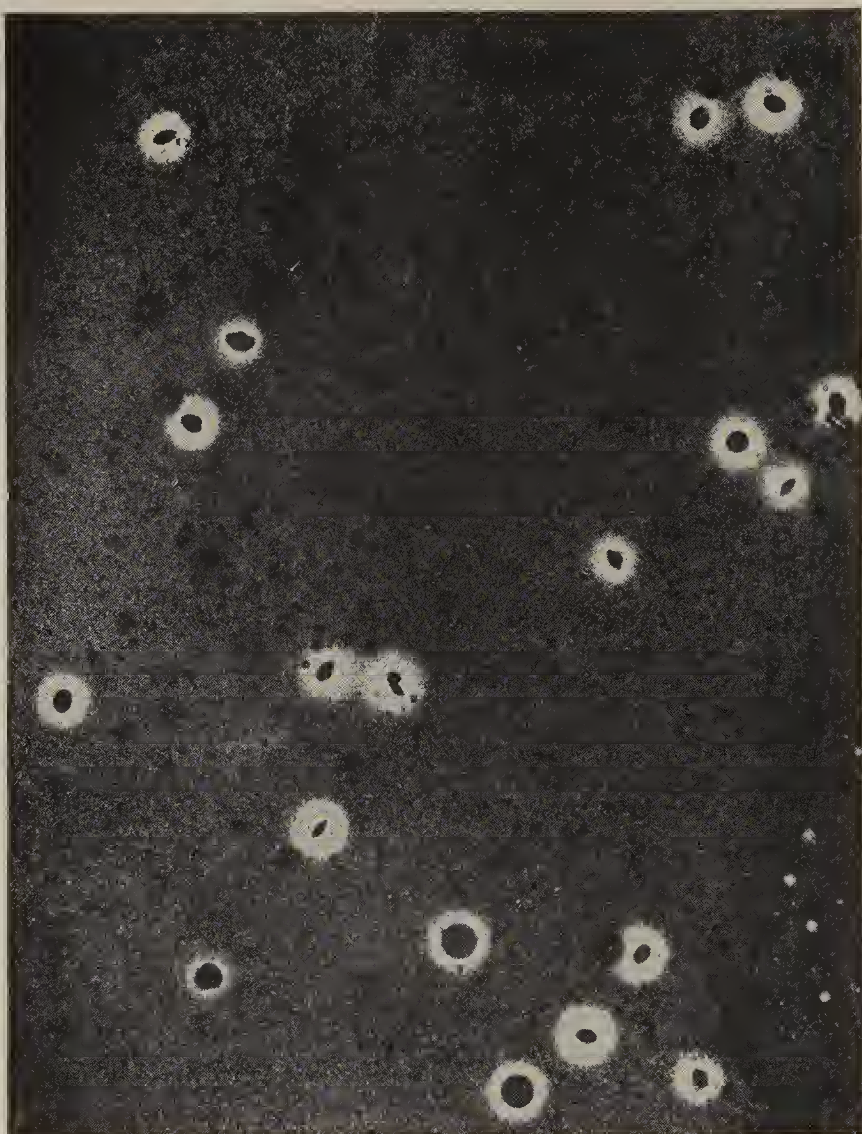


FIG. 43.—Solution of Calcium Carbonate by *Bact. lactis acidi.* (Orig. Northrup.)

REFERENCES

LÖHNIS, F.: Laboratory Methods in Agricultural Bacteriology, pp. 71–73.

VERNON: Intracellular Enzymes, p. 98.

EULER–POPE: General Chemistry of the Enzymes, pp. 31 and 58.

MARSHALL: Microbiology, 2d Ed., pp. 153, 154, 160, 164, 165, 378–381.

RAHN, O.: Tech. Bul. No. 10, Mich. Experiment Station, Fermenting Capacity of a Single Cell of *Bact. lactis acidi*, pp. 22–23.

EXERCISE 4. TO SHOW THAT ORGANIC ACIDS MAY SERVE AS A FOOD FOR SOME ORGANISMS

Apparatus. Two sterile 200 c.c. Erlenmeyer flasks; sterile 5 c.c. pipettes; 200 c.c. whey, soured by *Bact. lactis acidi.*

Cultures. *Oospora lactis.*
Mycoderma (pickle scum yeast).

Method. **1.** Titrate the acid liquid and record the titre.

2. Place 100 c.c. in each flask.

3. Heat for one hour, cool and inoculate each flask with one organism.

4. Titrate every two days from the time growth shows until the reaction becomes constant. *Always use sterile pipettes for obtaining a sample for titration.*

5. Plot curves on the same paper, using the same zero point.

6. Did you place the organism in its natural habitat? Will either of these organisms use another acid except that common to its habitat?

7. What is the chemical nature of this organic acid?

8. What has happened to the organic acid in question? Write the chemical equation showing this action.

9. What type of enzyme is concerned in the change which takes place?

10. State your results in detail and point out any conclusions to be drawn. Point out any practical applications that may be made.

REFERENCES

Lafar: Technical Mycology, English Ed., Vol. II, Part 2, pp. 417, 418, 452–455.

Laser: Biological Test for Butter. Zeitschrift für Hygiene (1891), Vol. X, p. 513.

Marshall: Microbiology, Second Edition, pp. 167, 168.

Northrup: Tech. Bul. No. 15, Michigan Experiment Station. The Influence of Certain Acid-destroying Yeasts upon Lactic Bacteria, pp. 5–7.

EXERCISE 5. TO DEMONSTRATE THE VARIATION IN FOOD REQUIREMENTS OF BACTERIA AND THEIR SELECTIVE POWER

Apparatus. Two tubes of sterile fermented agar; two sterile Petri dishes; potassium phosphate, di-basic; asparagin; peptone; ammonium sulphate; sodium nitrate; dextrose; lactose; saccharose.

Culture. *B. prodigiosus.*

Method. **1.** Melt the tubes of agar in the steam and, when cool but still liquid (about 40° C.), inoculate each heavily with *B. prodigiosus* and pour the plates. Allow to stand twenty-four hours at room temperature before proceeding. Is there any visible growth on the plate?

2. Divide each plate into five sectors; use the first four of the above chemicals on one plate and the last four on the second plate and leave a sector on each plate as a check.

3. Use a wax pencil to indicate the places of chemicals, which should be deposited in each sector and spread out in a very thin layer over the surface.

4. Use *very small* quantities of the chemicals and *be very careful not to scatter them* over the plate while conveying them to their proper places, otherwise the purpose of the experiment will be defeated.

5. Incubate at room temperature and examine the plate from day to day for growth.

Does the fermented agar support growth of itself? What explanation can you give for the action which occurs?

6. How is the variation in food requirements of *B. prodigiosus* shown? The selective action? Give another example of the demonstration of the selective action of bacteria. Which source of nitrogen is seemingly least available? Which most available? Why? Which carbohydrate is most easily digested? Which least? Why?

Beijerinck, knowing that agar in and of itself is a food

for but very few microorganisms, reasoned that this substance might be used for making solid synthetic media if it could be freed in some way from all traces of food materials. This he hoped to accomplish by allowing the agar to ferment spontaneously upon the addition of water.

In order that agar may not support microbial growth it must be allowed to ferment over a long period of time to exhaust every possible trace of food.

7. State your results in full, draw any conclusions that follow and point out the practical applications that may be made.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 151–153.
Fischer, A.: Structure and Functions of Bacteria, p. 115.

EXERCISE 6. TO DEMONSTRATE THE SPLITTING OF CARBOHYDRATES INTO ALCOHOL AND CO_2

Apparatus. Clean 375 c.c. Erlenmeyer flask fitted with one-hole rubber stopper containing a bent glass tube plugged at the end with cotton; two calcium chloride tubes; potash bulb; calcium chloride (small granules); potassium hydroxide solution (1 part KOH, 2 parts H_2O); rubber tubing for connecting up apparatus; 400 c.c. fractional distillation flask; thermometer; 250 c.c. 5% saccharose bouillon.

Culture. *Sacch. cerevisiæ.*

Method. **1.** Place the saccharose broth in the 375 c.c. flask, insert the rubber stopper and sterilize by the discontinuous method.

2. When sterile, inoculate with the yeast and connect the flask in " train " with a $CaCl_2$ tube (to remove moisture) a tared potash bulb (to take up CO_2) and a second $CaCl_2$ tube.

3. Place at 25° to 30° C. and allow to stand until no more gas evolves (about two weeks).

4. Test quantitatively for alcohol (distill off over 10 c.c. of liquid, measure the distillate and determine its specific gravity*) and estimate percentage of yield.

5. Weigh the potash bulb to find the amount of CO_2 given off. Does it correspond to the yield of alcohol? Explain. Are your results according to theory?

6. Write the chemical equation for each change, giving the specific enzymes concerned in each reaction. What types of enzymes are concerned?

7. Would alcohol be formed in bouillon containing no sugar? In a 5% aqueous solution of sugar? Why?

What fermentable substances are present in ordinary meat bouillon?

8. State your results in full and draw any conclusions warranted. What practical applications may be made of the above?

REFERENCES

Marshall: Microbiology, Second Edition, pp. 194–200.
Lafar: Technical Mycology, Vol. II, Part II, pp. 473–481, 511–515.
Hawk: Physiological Chemistry, Sixth Edition, pp. 369 and 479.

EXERCISE 7. TO DEMONSTRATE THE NECESSITY OF NITROGEN IN SOME FORM FOR MICROBIAL GROWTH

Apparatus. Four tubes each of:

Ordinary broth (organic nitrogen, soluble albumins and proteins).

Dunham's solution (organic nitrogen, soluble peptone, no albumen).

Uschinsky's asparagin medium (organic nitrogen, protein-free).

Cohn's solution (inorganic nitrogen combined with organic acid).

* Table for determining per cent of alcohol from specific gravity in Sadtler's Industrial Organic Chemistry (1912), pp. 579–584.

Winogradski's medium for nitrate formation (inorganic nitrogen combined with an inorganic acid).

Winogradski's medium for symbiotic nitrogen-fixation (nitrogen-free).

Cultures. *B. subtilis; Ps. radicicola; Aspergillus niger; Sacch. ellipsoideus.*

Method. **1.** Inoculate heavily a tube of each medium with *Ps. radicicola.* Proceed likewise with the three remaining organisms.

2. Record the growth at the end of five days. What conclusions may be drawn?

3. Compare the formulæ of the different media given above. *Organic nitrogen is present in the radical* NH_2, *inorganic,* NH_4.

4. What is the explanation for the growth of one organism and not another on a certain medium?

Do the organisms obtain their carbon from an organic or an inorganic compound in each case? Is organic or inorganic nitrogen the most available in each case? What is the value of such a medium? Why are other chemicals added besides the main nutrient?

Why is distilled water used in all these media? To what does the term " auxanography " refer?

5. Give your results in detail and draw any conclusions warranted. What practical applications may be made of the above?

REFERENCES

Marshall: Microbiology, Second Edition, pp. 151, 152 and 312.
Lafar: Technical Mycology, Vol. I, pp. 375, 468.
Löhnis: Laboratory Methods in Agricultural Bacteriology, p. 58.

EXERCISE 8. TO DEMONSTRATE THE PRODUCTION OF H_2S BY BACTERIA

Apparatus. Three tubes ordinary gelatin; tube ordinary agar; sterile Petri dish; lead carbonate, 0.1 gm.

Cultures. *B. coli communis; B. mycoides; B. mesentericus vulgatus. Bact. abortus.*

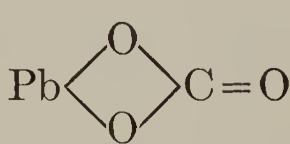
Method. 1. Make stabs of all organisms in gelatin and place these at a temperature not exceeding 20° C.

2. Melt a tube of agar and while hot add 0.1 gm. of lead carbonate to the tube and mix well by rolling it vigorously between the hands (avoid air bubbles).

3. Pour into the sterile Petri dish and when cold make a streak (2.5 cm. long and 3 cm. apart) of each organism on the plate in the order named. Invert and place at 25° C.

4. Examine the gelatin stabs from day to day for liquefaction; examine the plate culture at the same time. Note the action on lead carbonate (*Beijerinck's test*).

5. Write chemical equations for the action of sulphureted hydrogen on lead carbonate.

Lead carbonate =


6. Is there any relation between the power of organisms to liquefy gelatin and to produce "lead-blackening" sulphur? Explain.

From what compounds is the H_2S produced in this experiment? What type of organisms can be detected by this test? Where do they occur in the largest numbers in nature?

Which of the ordinary laboratory media offer the greatest source from which this gas may be produced? Explain.

By what other means may H_2S production by bacteria be demonstrated?

7. Give your results in full. Draw any conclusions

possible and point out any practical applications that may be made.

REFERENCES

LAFAR: Technical Mycology, Vol. II., Part 2, pp. 558–560.
MARSHALL: Microbiology, 2d Ed., pp. 172, 358–360.
LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 42, 116.
EYRE: Bacteriological Technic, 2d Ed., 1913, pp. 290–291.

EXERCISE 9. THE EFFECT OF PHYSICAL AND CHEMICAL AGENCIES ON MICROBIAL PIGMENT AND THEIR FORMATION

Apparatus. Seven tubes of gelatin; twenty-one dextrose agar slants +1.5 normal; eight tubes of plain milk, sterile; hydrochloric acid; sodium hydroxide; chloroform; ether; benzol; carbon bisulphide; alcohol; litmus paper: clean slide; small funnel.

Cultures. *Ps. pyocyanea; B. violaceus; B. prodigiosus; Sarcina lutea; Torula rosea; B. cyanogenus; Bact. lactis acidi.*

Method. *A. Effect of temperature on pigment formation.*

1. Make two dextrose agar streak cultures of each organism.

2. Place the cultures in duplicate at 25° C. and 37° C.

3. Examine every day or so and at the end of a week record the degree of pigment formation by ++, +, +−, −. *Brightness of pigment formation* should be considered in all cases, *not the amount of growth.*

4. Where is the pigment seen macroscopically in each case? Explain.

Does temperature have any influence on pigment formation? Does this correspond in each case with that of the natural habitat of the organism?

How do these several pigments differ? Of what importance is pigment production?

B. Relation of air to pigment formation.

Make gelatin stabs of all organisms and keep at or below 20° C. Note the place of pigment formation. Explain.

C. Relation of light to pigment formation.

Make two streaks of *B. prodigiosus.* Place one in *bright sunlight,* keep the other in the dark. Explain the results.

D. Effect of chemicals on pigment.

1. To one of the brightest pigmented cultures of *B. prodigiosus,* add 10 c.c. of 95% alcohol and shake vigorously. Alcohol dissolves the pigment.

2. Pour off into a flask and allow to settle. Filter.

3. Divide the clear filtrate into four parts.

To one, add a drop or two of HCl; note the result and explain. To the second add a drop or two of NaOH; note the result and explain.

Place the third in *bright sunlight* and note what happens.

Place a few drops of the fourth portion on a clean slide and allow to evaporate slowly. Examine crystals under microscope and draw. What are these crystals? Explain.

E. Solubility of pigment.

1. Make five dextrose agar streak cultures of *B. prodigiosus* and, when *well pigmented,* try the solubility of the pigment in (*a*) water, (*b*) chloroform, (*c*) ether, (*d*) benzol, (*e*) carbon bisulphide. Results?

2. Are any of the different bacterial pigments formed, water-soluble? What is the simplest method for determining whether the pigment produced by an organism is water-soluble?

F. Blue milk and "bloody" milk.

1. Inoculate milk tubes as follows:

Organism.	Alone.	+ Bact. lactis acidi.
Torula rosea...........		
B. cyanogenus.........		
B. prodigiosus.........		
Control..............		

and keep at 25° C. along with uninoculated control. Observe daily.

2. At the end of seven days test the reaction of each. Is there any relation between the reaction and pigment production?

3. What conditions are conducive to the formation of red milk? of blue milk?

How would you describe and explain "bloody" milk as produced by microorganisms to anyone unfamiliar with the phenomenon? How differentiated from true bloody milk?

4. State all results in full. Draw any conclusions warranted and point out the practical applications that may be made.

REFERENCES

LAFAR: Technical Mycology, Vol. I, pp. 137, 140, 149, and 157.
THRESH: Examination of Waters and Water Supplies, Second Edition, pp. 43–44.
MARSHALL: Microbiology, Second Edition, pp. 175–177.
CONN: Agricultural Bacteriology, pp. 156–157.

EXERCISE 10. TO ILLUSTRATE ONE OF THE PHYSICAL PRODUCTS OF METABOLISM

Apparatus. Three gelatin slants (−2.0% normal, 3% salt); rubber stopper to fit one of the gelatin tubes.

Culture. *Ps. lucifera* or some actively phosphorescing organism.

Method. **1.** Make a streak culture of the above organism upon each of the gelatin slants.

2. With one of the cultures, boil a rubber stopper and insert in place of the cotton plug.

3. Place the stoppered culture and a second one (cotton-plugged) at 20° C., the third cotton-plugged culture at 5°–10° C.

4. Examine in the light and in the dark after twenty-four and forty-eight hours. Compare (in the dark) the two cultures at 20° C.; if there is a *marked* difference, loosen the rubber stopper and note what happens.

5. If there is no immediate result from loosening the

stopper, replace the stopper with a sterile cotton plug and note both cultures after twenty-four hours. What occurs in either case?

6. Which is the better temperature for the growth of this organism? Can you suggest a reason why? What is the natural habitat of this type of organism? Of what importance are they?

What would you conclude regarding the respiration of phosphorescent bacteria? What term is applied to bacteria exhibiting this phenomenon? Of what importance is this phenomenon?

7. State your results in full, and draw any conclusions. What practical application of the above may be made?

REFERENCES

Lafar: Technical Mycology, Vol. I, pp. 160, 161.
Marshall: Microbiology, Second Edition, p. 188.
Fischer, A.: Structure and Functions of Bacteria (1900), pp. 63–64.

ENZYMES: CLASSIFICATIONS AND REACTIONS

Enzymes can be classified in several different ways:

I. According to their place of activity as *endo-enzymes* (intracellular) or *exo-enzymes* (extracellular);

II. According to the type of food substance acted upon, as *proteolytic* (protein-digesting), *lipolytic* (fat-digesting), enzymes attacking carbohydrates, etc.;

III. The most satisfactory and inclusive classification is that denoting the chemical reactions produced by the enzyme during its activity. Enzymes may thus be called:

1. *Hydrolytic*, the addition of one of more molecules of water to the molecule of the substance acted upon.

2. *Reducing-oxidizing*, causing a reduction of one part of the molecule, the oxygen so obtained being used probably to oxidize another portion of the molecule. A number of the

enzymes of this class causes this rearrangement, splitting the molecule *without the addition to* or *subtraction from any elements therein.*

3. *Oxidizing,* the addition of oxygen to (or the subtraction of hydrogen from) the molecule of the substance acted upon.

4. *Reducing,* the subtraction of oxygen from (or the addition of hydrogen to) the molecule of the substance acted upon. The reducing enzymes are the only class of enzymes in the above classification acting upon inorganic compounds; some organic compounds are also acted upon, viz., litmus, methylen blue, etc.

5. *Coagulating,* unknown processes accompanied by coagulation. Enzymes whose actions are not so well known are those producing syntheses, isomers, acting anaerobically, etc.

Note. *Euler's suggestion that the names of enzymes be formed from the compound acted upon, by suffixing "-ase," will be adhered to in all subsequent study of enzymes, the suffix "-lytic" for the adjective, and the suffix "-ese" for synthesizing enzymes.*

Bayliss has suggested the ending "-clastic" for the adjective, criticizing the ending "-lytic" because the definition of "electrolytic," which must be granted priority, implies action by the agent rather than upon the substance indicated by the term. He also questions the existence of Euler's "synthesizing enzymes."

CLASSIFICATION OF ENZYMES

I. Hydrolytic Enzymes of:

A. Carbohydrates, including Glucosides, *carbohydrases*—general term.

1. Polysaccharides. $(C_6H_{10}O_5)x$.
   - *a.* Celluloses: *cellulases*—general term.
   - *b.* Hemicelluloses: *cytases*—general term.
   - *c.* Starches, insoluble and soluble: *amylases,* (ptyalin, diastase)—general term.
   - *d.* Glycogens: *glycogenases*—general term.
   - *e.* Dextrins: *dextrinases*—general term.

2. Disaccharides. $C_{12}H_{22}O_{11}$.
   a. Saccharose: *sucrase* (invertase, invertin)—specific term.
   b. Lactose: *lactase*—specific term.
   c. Maltose: *maltase*—specific term.
3. Glucosides: *glucosidases*—general term.
   a. Amygdalin: *amygdalase* (emulsin, synaptase)—specific term.
   b. Tannin (digallic acid): *tannase*—specific term.
4. Pentoses: $(C_5H_{10}O_5)x$.
   a. Pectoses: *pectases*—general term.

B. Esters: *esterases*—general term.
1. Fats: *lipases* (steapsin)—general term.
   a. Stearin: *stearinase*—specific term.

C. Proteins: *proteinases* or *carbamases*—general term.
1. Protein-digesting.
   a. Proteins broken down to proteoses and peptones: *peptase* (pepsin or acid-proteinase)—general term.
   b. Proteins broken down further to polypeptids and occasionally to *a*-amino acids with a trace of ammonia: *tryptase* (trypsin or alkali-proteinase)—general term.
   c. Proteoses, peptones, polypeptids and protamins broken down completely to a-amino acids with a trace of ammonia: *ereptase* (erepsin, protease)—general term.

D. Acid amides (urea): *amidases*—general term, to form:
1. Ammonium carbonate: *urease*—specific term.

II. Reducing-oxidizing enzymes: *oxyhydrases*—general term, of:

A. Carbohydrates (d-hexoses) $C_6H_{12}O_6$ to form:
1. Alcohol, ethyl and carbon dioxide: *zymase*—general term.
   a. Dextrose: *dextro-zymase*—specific term.
   b. Levulose: *levulo-zymase*—specific term.

c. Galactose: *galacto-zymase*—specific term.

2. Lactic acid: *lactic acid bacteria zymase*—specific term.

III. Oxidizing enzymes: *oxidases*—general term, of:

A. Ethyl alcohol: *alcoholase* (alcoholoxidase, vinegar-oxidase)—specific term.

B. Organic acids:

1. Lactic acid: *lactacidase*—specific term.
2. Acetic acid: *acetacidase*—specific term.

C. Tyrosin: *tyrosinase*—specific term.

IV. Reducing enzymes: *reductases*—general term, of:

A. Hydrogen peroxide:

1. *Catalase*—specific term, free oxygen liberated.
2. *Peroxidase*—specific term, transference of oxygen.

B. Organic dyes to leuco-compounds.

1. Methylen blue, litmus, azolitmin, indigo, etc.: *methylen-blue reductase*, etc.—specific term.
2. Methylen blue in the presence of formaldehyde (Schardinger's reaction): *perhydridase*—specific term.

C. Sulphur to H_2S: *sulphur reductase*—specific term.

D. Nitrates to nitrites, nitrates to NH_3, etc.: *nitrate–, nitrite–reductase*, etc.

V. Coagulating enzymes.

A. Protein-coagulating.

1. Casein:
   a. Of cow's milk: *caseinase* (rennin, rennet, chymosin)—specific term.
   b. Of human milk: *parachymosin*—specific term.
2. Fibrin of blood: *thrombase* (thrombin)—specific term.

B. Carbohydrate-coagulating.

1. Pectin: *pectinase*—specific term.

ENZYMIC REACTIONS OF WELL KNOWN FERMENTATION PROCESSES

A. Beer or bread fermentation by *Sacch. cerevisiæ.*

I. starch + water + hydrolytic enzyme = maltose.

$2(C_6H_{10}O_5) + xH_2O +$ *amylase* (from malt) $= xC_{12}H_{22}O_{11}$.

II. (*a*) maltose + water + hydrolytic enzyme (from yeast) = 2 mols. dextrose.

$C_{12}H_{22}O_{11} + H_2O +$ *maltase* $= 2C_6H_{12}O_6$.

III. dextrose + enzyme (from yeast) = alcohol + carbon dioxide.

$C_6H_{12}O_6 +$ *yeast zymase* $= 2CH_3CH_2OH + 2CO_2$.

This same yeast, and other yeasts also, can ferment saccharose (cane sugar) corresponding to II above, as follows:

II. (*b*) saccharose + water + hydrolytic enzyme (from yeast) = d-dextrose + d-levulose.

$C_{12}H_{22}O_{11} + H_2O +$ *sucrase* $= C_6H_{12}O_6 + C_6H_{12}O_6$.

Both of these simple sugars can be fermented to alcohol and CO_2 according to III above.

Comparatively few yeasts can attack *lactose* (milk sugar), e.g., *Sacch. kefir* (p. 369, Marshall), *Sacch. fragilis*, *Sacch. tyricola*, etc. (See p. 106, Guilliermond's Les Levures.) The reactions are similar to II (*a*) above, as follows:

(*c*) lactose + water + hydrolytic enzyme (from yeast) = d-dextrose + d-galactose.

$C_{12}H_{22}O_{11} + H_2O +$ *lactase* $= C_6H_{12}O_6 + C_6H_{12}O_6$.

Both simple sugars are changed to alcohol and CO_2 according to III above.

B. Glucoside decomposition, by molds, bacteria and yeasts.

General reaction,

glucoside + water + hydrolytic enzyme = sugar + aldehydes, acids, etc.

Specific reaction,

amygdalin + water + *emulsin* = dextrose + benzaldehyde + hydrocyanic acid.

$C_{20}H_{27}O_{11}N + 2H_2O +$ *emulsin** $= 2C_6H_{12}O_6 + C_6H_5CHO + HCN$.

* Emulsin is a mixture of four different enzymes.

C. Fat decomposition, by a few molds, yeasts and bacteria. Only microbial method of fat decomposition.

General reaction,

fat + water + hydrolytic enzyme = fatty acid + glycerin.

Specific reaction,

stearin + 3 mols. water + *stearinase* = 3 mols. stearic acid + glycerin.

$$\begin{array}{llll} C_{17}H_{35}CO \cdot O\text{–}CH_2 & H_2O & C_{17}H_{35}COOH & CH_2OH \\ \qquad\qquad\quad | & & & \ \ | \\ C_{17}H_{35}CO \cdot O\text{–}CH \ + & H_2O = & C_{17}H_{35}COOH \ + & CHOH \\ \qquad\qquad\quad | & & & \ \ | \\ C_{17}H_{35}CO \cdot O\text{–}CH_2 & H_2O & C_{17}H_{35}COOH & CH_2OH \end{array}$$

Lipases decompose more especially the natural fats, i.e., the glycerin esters of palmitic, stearic and oleic acids. Lipases from different sources differ markedly in reactions.

D. Vinegar fermentation.

In order that this fermentation may take place, alcohol must be present in the nutrient solution, either added artificially or as a product of fermentation. In the latter case, reactions I, II*a* and III, II*b* and III, or II*c* and III under *A* above must precede those of the vinegar fermentation.

Assuming that alcohol is present in the liquid in which the vinegar bacteria are growing, the reactions take place in two stages, as follows: (See p. 448, Marshall.)

I. ethyl alcohol + oxygen + oxidizing enzyme = acetaldehyde + water

$$CH_3CH_2OH + O + \textit{alcoholase} = CH_3CHO + H_2O.$$

II. acetaldehyde + oxygen + oxidizing enzyme = acetic acid

$$CH_3CHO + O + \textit{acetaldehydase} = CH_3COOH$$

If there is not plenty of air present the oxidation may not become complete and small amounts of acetaldehyde may form, i.e., the reaction stops at the first stage.

If the initial percentage of alcohol is below 1 to 2% the vinegar bacteria will soon attack the acetic acid, oxidizing it completely to carbon dioxide and water, as follows:

III. acetic acid + oxygen + oxidizing enzyme = carbon dioxide + water

$$CH_3COOH + 4O + \textit{acetacidase} = 2CO_2 + 2H_2O.$$

This cannot take place, however, if above 10 to 12% acetic acid is present, as this amount is antiseptic to the vinegar bacteria. (See pp. 450–451, Marshall.)

E. Organic acid decomposition, by acidophile organisms (organisms of the *Oidium* and *Mycoderma* type).

lactic acid + oxygen + oxidizing enzyme = carbon dioxide + water
$CH_3CHOHCOOH + 6O +$ *lactacidase* $= 3CO_2 + 3H_2O$.

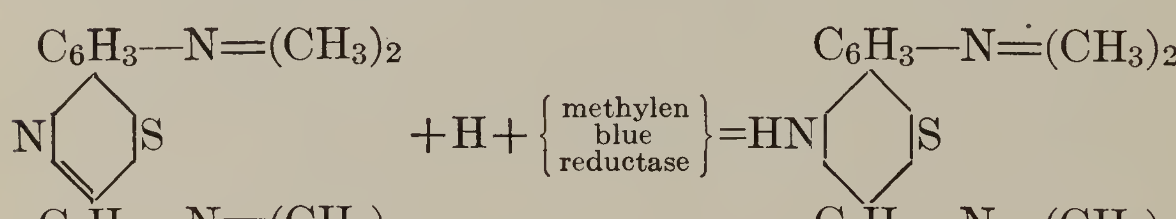
The destruction by oxidation of acetic acid by the acetic bacteria is given under *D* above.

Nearly all organic acids are decomposed in a similar manner, *by total combustion.*

F. Reactions of reductases.

hydrogen peroxide − oxygen + reducing enzyme = water + oxygen.
$H_2O_2 - O +$ *catalase* $= H_2O + O$.

methylen blue + hydrogen + *methylen blue reductase* = leuco-base of methylen blue.



G. Lactic acid fermentation, produced in milk by *Bact. lactis acidi.*

lactose + water + hydrolytic enzyme = d-dextrose + d-galactose.
$C_{12}H_{22}O_{11} + H_2O +$ *lactase* $= C_6H_{12}O_6 + C_6H_{12}O_6$.

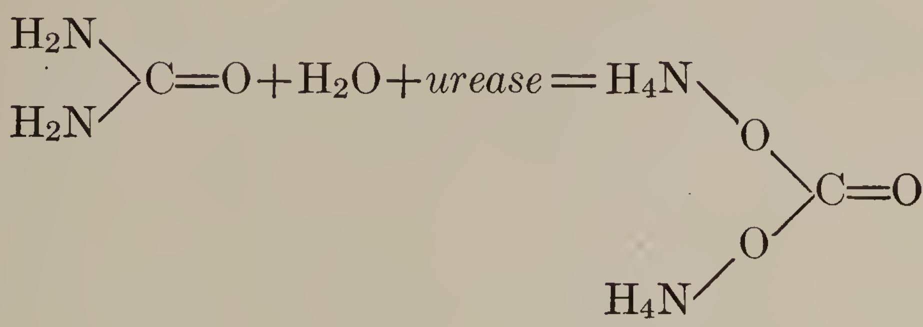
$\left.\begin{array}{l}\text{dextrose}\\ \text{galactose}\end{array}\right\} + \text{enzyme} = \text{lactic acid.}$

$2C_6H_{12}O_6 +$ *lactic acid bacteria zymase* $= 4CH_3CHOHCOOH$.

Bact. lactis acidi will ferment a nutrient solution containing only a simple sugar, e.g., dextrose, the reaction then being according to the second equation.

H. Urea fermentation, by urea bacteria.

Urea + water + hydrolytic endo-enzyme = ammonium carbonate.



EXERCISE 11. A COMPARISON OF ACID AND RENNET CURDS

Apparatus. Three 200 c.c. flasks containing 100 c.c. each of sterile skim milk; 200 c.c. *fresh* skim milk; small funnel; eighteen large test tubes; absorbent cotton; 10% lactic acid; 5% phenol.

Cultures. *B. prodigiosus; Bact. lactis acidi; B. megaterium.*

Method. **1.** Inoculate flasks containing 100 c.c. sterile mill- with *B. prodigiosus, Bact. lactis acidi* and *B. megaterium.*

2. Place about 30 c.c. fresh skim milk in a 200 c.c. flask.

3. Add 10% lactic acid *drop by drop,* shaking constantly.

4. When the *first finely divided* curd appears, titrate. What *degree* and *percent* of acid were necessary to curdle the milk?

5. Titrate fresh skim milk.

6. Prepare 150 c.c. of 0.5% phenol milk (by adding 15 c.c. of 5% phenol to 135 c.c. of milk and sterilize). Mix well and titrate again. Is the acidity of the milk increased perceptibly by the addition of phenol?

7. As soon as curd appears in inoculated flasks, titrate. Determine the *degree* and *percent* of acidity present.

8. Allow the cultures to develop several days until decided proteolysis is evident. Then titrate again.

9. Add 10 c.c. of xylol to each flask, shake well and filter each culture through absorbent cotton (a small piece in small funnel); 15 c.c. of each filtrate is necessary.

10. Mix the filtrate from each culture with phenol milk in the following proportions:

1. ....0.5 c.c. filtrate+9.5 c.c. phenol milk.
2. ....1.0 c.c. filtrate+9.0 c.c. phenol milk.
3. ....2.0 c.c. filtrate+8.0 c.c. phenol milk.
4. ....3.0 c.c. filtrate+7.0 c.c. phenol milk.
5. ....4.0 c.c. filtrate+6.0 c.c. phenol milk.
6. ....Heat 4 c.c. of filtrate only, in steam for fifteen minutes. After cooling, add 6 c.c. phenol milk.

Shake these mixtures well and incubate at 37° C.

11. Record the time necessary for coagulation in each case. Why do not all tubes change alike? Explain.

12. Can corrosive sublimate be used to replace phenol in this experiment? Explain.

What types of enzymes are concerned in these changes?

Are these intra- or extra-cellular in each case? Will the place of occurrence of the enzymes explain the action taking place in the different sets of tubes?

What enzymes produce each type of curd?

What are the differences between an acid and a rennet curd?

Which type is produced by each of the organisms used?

What effect has heat upon enzymes?

13. Give results in full and draw any conclusions permitted. Point out any practical applications of the above.

REFERENCES

Lafar: Technical Mycology, Vol. I, pp. 240–244.
Bayliss: Nature of Enzymic Action, p. 37.
Euler: General Chemistry of the Enzymes, pp. 45–48, 58.
Marshall: Microbiology, Second Edition, p. 199.
Vernon: Intracellular Enzymes, pp. 220–221.
Cohnheim: Enzymes, pp. 29, 87–89.

EXERCISE 12. TO SHOW THE ACTION OF PROTEOLYTIC ENZYMES UPON GELATIN

Apparatus. Phenol, 0.5% solution (10 c.c. of 5.0% phenol+90 c.c. distilled H_2O); water bath and thermometer; gelatin, 7 gms.; 15 tubes sterile gelatin; formalin; xylol; 5 sterile 1 c.c. pipettes; centimeter scale.

Cultures. *B. ramosus; B. fluorescens; B. subtilis; B. mycoides; B. prodigiosus.*

Method. 1. Make two gelatin stab cultures of each organism and when *nearly all* liquefied proceed with the experiment.

2. Dilute the 5% phenol to 0.5% as above, with distilled water.

3. Add 7 gms. gelatin, dissolving by heating not over 70° C. Neutralize carefully.

What is the source of acid in phenol gelatin? Why is the gelatin neutralized?

4. Select five test tubes having the same diameter. Fill each half full. Solidify in an upright position.

5. Shake each of the liquefied cultures with 3 to 4 c.c. of xylol.

6. After one hour, add 1 c.c. of the clear supernatant xylol solution of each culture to a tube each of solid phenol gelatin and of ordinary gelatin. With a blue pencil, mark the surface of the solid gelatin.

7. Examine the tubes daily. Is there any evidence of growth? Of liquefaction? If liquefaction is noted, measure its progress in millimeters.

8. Save the original cultures with which the xylol has been shaken. Is there any evidence of further growth?

9. If 1 c.c. of a liquefied gelatin culture of a liquefying organism were added to a tube of solid ordinary gelatin, what would happen? What would result if it were added to a tube of solid phenol gelatin? Explain.

10. What action does the xylol have? How can you

prove that xylol has this action? What other chemicals could be used in place of xylol?

11. What is the object of adding phenol to the gelatin? Would 5% phenol serve the same purpose? Give reason for answer. What other chemicals could be used in place of phenol? Why? What chemicals could not be used in place of phenol? Why? How else may *pure enzyme action* be demonstrated?

12. Add 5 drops of 40% formaldehyde (formalin) to each tube of the duplicate liquefied gelatin cultures and note whether they become solid again in a few days. *Explain the action.*

13. Give your results in full and draw any conclusions possible. What practical applications of the above may be made?

REFERENCES

Bayliss: Nature of Enzymic Action, p. 37.

Marshall, C. E.: Microbiology, Second Edition, pp. 169, 194, 197–199, 201, 276, 277 and 320.

Euler, Hans: General Chemistry of the Enzymes (1912), pp. 115–123.

Vernon, H. M.: Intracellular Enzymes (1909), pp. 215–220.

EXERCISE 13. TO SHOW THE ACTION OF PROTEOLYTIC ENZYMES UPON CASEIN

Apparatus. Five tubes of sterile milk; two tubes nutrient agar; sterile 5 c.c. pipettes; two sterile Petri dishes.

Cultures. *Bact. lactis acidi; B. ramosus; B. coli; B. violaceus.*

Method. **1.** Warm the milk (40–45° C.).

2. Place 2 c.c. in each sterile Petri dish and pour one tube of melted agar upon it, mix thoroughly by carefully tilting.

3. When solid, make parallel streaks with *Bact. lactis acidi* and *B. ramosus* upon one and of *B. coli* and *B. violaceus* upon the other. Transfer cultures to litmus milk also.

4. Examine streak cultures *every* day for evidences of proteolysis. Make drawings and compare the rate of action of the different bacteria. Compare streak with milk cultures.

5. Is there any relation between the power of enzymes to liquefy gelatin and their ability to dissolve casein? What type of proteolytic enzyme dissolves casein?

6. Give your results in detail. Draw any conclusions which follow and point out any practical applications that may be made.

REFERENCES

Marshall, C. E.: Microbiology, Second Edition, pp. 197–199, 428, 430.
Hastings: The action of various classes of bacteria on casein as shown by milk agar plates. Cent. f. Bakt., II Abt., Bd. 12 (1904) p. 590.

EXERCISE 14. TO SHOW THE ACTION OF ENZYMES UPON STARCH

Apparatus. Three sterile Petri dishes; three test tubes; soluble starch; three tubes sterile agar; Lugol's iodin solution.

Cultures. Soil for inoculation.

Method. 1. Place 0.1 gm. of soluble starch in each test tube, plug and sterilize in the hot air sterilizer.

2. To each tube of starch add one tube of melted agar.

3. When at the correct temperature (40°–45° C.) inoculate one tube with one loopful of soil. (State type used.) Inoculate the second from the first, etc., then plate all three dilutions.

4. When the colonies are well developed, pour iodin solution on the plate and note any clearing around the colonies. What does this indicate?

5. Examine microscopically different types of colonies attacking starch. Are they molds, yeasts, or bacteria? Which type predominates on your plates?

6. What enzymes are concerned? Give specific action. How is pure enzymic action demonstrated?

7. Write the theoretical chemical equation. What is soluble starch?

8. What is the value of such microbial action in soil? Where are starch-digesting microorganisms present in nature? Of what importance?

9. State results in full and draw any conclusions. Point out any practical applications of the above.

REFERENCES

Bayliss: Nature of Enzymic Action, pp. 25, 113.

Marshall, C. E.: Microbiology, Second Edition, pp. 151, 166, 167, 315–318 and 555.

Euler, Hans: General Chemistry of the Enzymes, pp. 1 –15 *et al.*

Hawk, Philip B: Physiological Chemistry, Sixth Edition, pp. 10, 43–45, 57, 59, 60.

Sadtler, S. P.: Industrial Organic Chemistry, p. 186.

Lafar: Technical Mycology, Vol. II, Part 2, pp. 351–353.

EXERCISE 15. TO SHOW THE ACTION OF REDUCING ENZYMES

Apparatus. Petri dish; medium fine sand; sulphur; cake of Fleischmann's compressed yeast, *fresh* (obtain this yourself); small mortar and pestle; lead acetate paper.

Method. 1. Thoroughly grind the sulphur, sand and yeast cake in a small mortar.

2. Place the contents of the mortar in a covered dish with a piece of moistened lead acetate paper. What odors are noted?

3. What reaction is demonstrated by the lead acetate paper? What reactions are taking place? Give a chemical equation which will cover the final changes. May other enzymes be released from the yeast cells during the process of maceration? If so, what enzymes?

What names are applied to the specific enzyme acting on sulphur and to the class to which it belongs? Where does this action occur in nature?

This enzymic action was first observed in 1888 by a Frenchman, J. de Reypailhade, who found that the alcoholic extract of yeast would convert elementary sulphur into sulphuretted hydrogen.

4. Give all results in full and draw any conclusions permissible. What practical applications may be made of the above?

REFERENCES

MARSHALL, C E.: Microbiology, Second Edition, pp. 194, 202, 203, 360.
LAFAR, F.: Technical Mycology, Vol. II, Part II, pp. 558–560.
KRUSE, W.: Allgemeine Mikrobiologie, pp. 652–655.

EXERCISE 16. TO SHOW THE ACTION OF THE ENZYME CATALASE

Apparatus. Four fermentation tubes of nutrient broth (sterile); hydrogen peroxide (full strength).

Cultures. *B. coli; B. subtilis; B. mycoides; Bact. lactis acidi.*

Method. **1.** Inoculate the fermentation tubes.

2. After growth is well started, add 1 c.c. of hydrogen peroxide to each tube and mix well.

3. After the tubes have stood for half an hour measure the gas formed. Compare your results with those of other students.

Note. If the bottle of H_2O_2 stands uncorked or in a warm place it decomposes very rapidly and the gas formed in the fermentation tubes will be much less than from a full strength solution.

4. What is the strength of commercial hydrogen peroxide?

Where else is catalase found? What is the type of action supposedly taking place? Write chemical equation showing the general reaction.

Have you ever observed the action of catalase produced in animal tissues? What is the difference between catalase and peroxidase?

5. State the results of your experiment in full and draw any conclusions permissible. Point out any practical applications that may be made.

REFERENCES

Bayliss: Nature of Enzymic Action, pp. 140–141.
Euler–Pope: General Chemistry of the Enzymes, pp. 65, 67–68.
Vernon: Intracellular Enzymes, pp. 127–132.
Löhnis, F.: Laboratory Methods in Agricultural Bacteriology, pp. 66–67, 79.
Marshall: Microbiology, Second Edition, pp. 194, 202 and 203.

EXERCISE 17. TO DEMONSTRATE THE OXIDIZING ENZYME OF VINEGAR BACTERIA

Apparatus. 200 c.c. fermented cider (or other fruit juice); sterile 375 c.c. Erlenmeyer flask; sterile 10 c.c. pipette; water bath; specific gravity bottle.

Culture. *Bact. aceti.*

Method. **1.** Place the cider in a sterile flask and heat in a water bath at 60° C. for one hour. Cool quickly. What is this process called?

2. Determine the specific gravity of the fermented cider.

3. Inoculate with a pure culture of *Bact. aceti* and titrate every three days until the titre is constant.

4. Plot the curve showing and explain the direction which the curve takes. What is taking place? Enzyme? Chemical equation?

5. Determine the specific gravity of the solution at the last titration. How does this compare with specific gravity of cider vinegar of legal standard? What is the legal standard for vinegar in this state? Can you explain why all vinegar does not come up to the legal standard?

6. Is it practicable to use pure cultures for preparing vinegar? How do various species of vinegar bacteria differ from one another?

Under what conditions will acetic fermentation set in "spontaneously?"

What raw materials will give rise to a vinegar by a normal acetic fermentation?

How does a scarcity of alcohol influence the amount of acid produced? An excess of alcohol?

How may vinegar be prepared artificially? How adulterated?

7. State the results of your experiment in detail and draw conclusions. Point out any practical applications that may be made.

REFERENCES

EULER–POPE: General Chemistry of the Enzymes, pp. 60–61.
MARSHALL: Microbiology, Second Edition, pp. 194, 202, 538–550.
SADTLER: Industrial Organic Chemistry, pp. 266–272.
LAFAR: Technical Mycology, Vol. I, pp. 394–399.
Circular on "Vinegar" prepared by Bacteriological Laboratory, East Lansing, Mich.

EXERCISE 18. TO DEMONSTRATE THE NECESSITY OF AN ACTIVATOR FOR THE ENZYMIC ACTION OF RENNET (FROM CALF'S STOMACH)

Apparatus. Four clean 200 c.c. Erlenmeyer flasks; sweet skim milk (not over +1.5% normal); rennet, fresh commercial; two 1 c.c. pipettes; sterile saturated solution of monobasic calcium phosphate ($Ca(H_2PO_4)_2+H_2O$) or 0.16 to 0.25% hydrochloric acid; water bath; thermometer.

Method. 1. Place about 150 c.c. of skim milk in each of two 200 c.c. flasks, plug these and sterilize them by the Tyndall method.

2. When ready to start the experiment, obtain 300 c.c. of fresh skim milk and place 150 c.c. of milk in each of the two remaining flasks.

3. Mark the fresh milk flasks Nos. 1 and 2; the sterilized milk flasks Nos. 3 and 4.

4. Place all four flasks in a water bath and heat the water to 35° C., *not higher*. (*Steam cannot be substituted.*)

5. Mark the flasks as follows:

Flask No. 1 = unheated milk + rennet.
" " 2 = unheated milk + calcium phosphate + rennet.
" " 3 = heated (sterilized) milk + rennet.
" " 4 = heated (sterilized) milk + rennet + calcium phosphate.

6. Add 1 c.c. of the calcium phosphate solution to one flask of fresh milk and mix. (Flask No. 2).

7. To each flask of milk add a drop of rennet, shake quickly, replace the flask in the water bath and leave for ten to twenty minutes without disturbing.

8. Add 1 c.c. of calcium phosphate solution to flask No. 4. Shake quickly, return the flask to the water bath and leave for ten to twenty minutes without disturbing. Observe. If no curd appears, set the flasks at 37° C. and observe after about twenty-four hours. What is the explanation for the phenomena occurring in this flask?

9. Observe the milk in all flasks for curdling. Which flasks of milk curdled? Why?

10. What are the various synonyms of "rennet?"

What is the specific action of this enzyme?

What is the source of the enzyme used? How prepared?

What living organisms produce coagulating enzymes?

Does the rennet produced by various bacteria require soluble calcium salts for an activator? How would you determine this?

What is an activator? To what property of an activator is its action attributed? What are the different classes of activators? Do all enzymes require activators?

11. Give all results in full and draw any conclusions possible. What practical applications of the above may be made?

REFERENCES

Lafar: Technical Mycology, Vol. I, pp. 240–243.
Bayliss: Nature of Enzymic Action, pp. 120–121, 132–133.
Richmond: Dairy Chemistry, p. 301.
Euler–Pope: General Chemistry of the Enzymes, pp. 94, 106–109.

EXERCISE 19. EFFECT OF CONCENTRATED SOLUTIONS UPON MICROORGANISMS

Apparatus. 750 c.c. nutrient broth; gelatin; salt; dextrose; saccharose; five 10 c.c. pipettes; 100 c.c. graduate.

Cultures. *Mycoderma; B. coli; M. varians; Sacch. cerevisiæ; Penicillium; B. prodigiosus.*

Method. 1. Make up four tubes each of the following concentrations:

Electrolytes: sodium chloride, 5%, 10%, 15%, 20%, 25%.

Non-electrolytes: dextrose and *saccharose,* 30%, 45%, 60%, 75%, respectively.

Colloids: gelatin, 5%, 10%, 30%, 50%.

2. With the exception of the gelatin the separate weighing out for each concentration can be avoided by using the following method of mixing, with the stock solution containing 50% or 75% of the substance under study:

(*a*) Weigh out the correct quantity of material and place in a graduate.

(*b*) Fill the graduate to the 125 c.c. mark with nutrient broth. Place the hand over the mouth of the graduate and shake until solution is complete. If necessary, fill to the mark again with broth. *For example:* dissolve 31.25 grams of salt in about 90 c.c. broth, fill the graduate to 125 c.c. with broth. This will give a 25% solution. Mix this salt broth with common broth in the following proportions, by means of pipettes:

Salt broth.		Plain broth.	Salt content of mixture.
2 c.c.	+	8 c.c.	5%
4 c.c.	+	6 c.c.	10%
6 c.c.	+	4 c.c.	15%
8 c.c.	+	2 c.c.	20%
10 c.c.	+	0 c.c.	25%

Broth will give a precipitate after heating with salt, consequently each salt broth mixture has to be filtered separately after heating. What is this precipitated material?

Note 1. The stock solution of dextrose is best prepared by adding to 93.75 gms. dextrose, 75 c.c. of broth, heating the mixture in the steam until dissolved and then making up to 125 c.c. with broth. The sugar solutions may have to be filtered also.

Note 2. As it is a difficult procedure to make up as high concentrations of gelatin as 30% and 50% with any degree of ease and accuracy, gelatin prepared according to the following procedure will serve to illustrate the point of the exercise.

With a blue pencil, mark the 10 c.c. level on each of sixteen tubes. To make up 5% gelatin, place 0.5 gm. gelatin in each of four tubes and make up to the 10 c.c. mark with broth. Proceed similarly with the remaining concentrations. After heating once, mix well with a sterile platinum loop.

Gelatin is practically the only colloid that can be obtained in solutions concentrated enough for this experiment (up to 70%). Great care must be taken to avoid the condensation of moisture on the sides of the test tubes or flask, because this moisture will reduce the concentration of the surface gelatin and thus cause incorrect data.

3. Sterilize the tubes by the intermittent method.

4. Inoculate *heavily* one tube of each concentration of the salt with *Mycoderma*, one with *B. coli* and one with *M. varians*. Inoculate tubes of each concentration of dextrose, saccharose and gelatin with *Penicillium*, *Sacch. cerevisiæ*, and *B. prodigiosus*, leaving a tube of each concentration uninoculated for control.

Note. The inoculation must be heavy, because experience teaches that a small inoculum is sometimes not sufficient to secure growth.

5. Note and tabulate the growth after seven days.

6. *Reinoculate* the lowest concentration of each set that does not show growth from the highest of the same set that does grow, e.g., if *Penicillium* grows at 45% dextrose but not at 60%, inoculate 60% from 45%. If it now grows, what is indicated? Is there not plenty of water and food material present? Explain your results.

Does the natural habitat or food requirements of each organism explain in any way the action occurring?

7. What is meant by osmotic pressure? Electrolyte? Colloid? What is known of the relative osmotic pressure

of electrolytes, non-electrolytes and colloids? How are these differences explained? Is the relative preserving power of these different substances according to the molecular weight theory? Explain.

Why is a large inoculum more apt to insure growth than a small inoculum?

Note. Salt-resisting organisms can be secured by plating in agar containing 10 to 15% of salt, from butter, brine pickles, salt pork, salt fish, and other salted food. Sugar-resisting organisms can be obtained similarly.

8. State the results of your experiment in full and draw conclusions. Point out any practical applications that may be made.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 207–212.
Fischer, A.: Structure and Functions of Bacteria, pp. 5, 8–9.

EXERCISE 20. THE EFFECT OF DESICCATION UPON BACTERIA

Apparatus. Four sterile cover-glasses; four sterile Esmarch dishes; potato knife; eighteen tubes of sterile broth.

Cultures. *B. violaceus* (non-spore-producing, non-slime-forming); slimy milk bacillus (non-spore-producing, slime-forming); *B. subtilis* (spore-producing, non-slime-forming); meat bacillus (spore-producing, slime-forming).

Method. **1.** Using a platinum needle, smear one cover-glass thickly with a culture of *B. violaceus*, the second with the slimy milk bacillus, the third with the spore-former (*B. subtilis*) and the fourth with the spore and slime producing meat bacillus.

2. Place each of these cover-glasses in separate sterile Esmarch dishes and break each into five or six small pieces with a sterile potato knife.

3. Transfer a piece of each cover-glass to a tube of nutrient broth after 1, 3, 7, 14, etc., days.

Stop transferring when you find that there is no growth in the test tube last inoculated.

4. What influence has the physical condition of the substrate upon which the microorganisms are dried upon their longevity? Illustrate.

What dried cultures of microorganisms have been used with success commercially? Without success? What other methods may be employed to demonstrate the effect of desiccation on microorganisms?

5. State all results in full and draw any conclusions. Point out any possible practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 211, 212, 218, 350–353, 411–414, 442–444, 450–456.

Smith, E. F.: Bacteria in Relation to Plant Diseases, Vol. I, pp. 70–71.

Eyre: Bacteriological Technic, 2d Ed., pp. 306–308.

EXERCISE 21. THE DETERMINATION OF THE OPTIMUM, MAXIMUM AND MINIMUM TEMPERATURE REQUIREMENTS FOR CERTAIN ORGANISMS

Apparatus. Sixteen tubes of dextrose broth.

Cultures. *Sacch. cerevisiœ; B. subtilis; Oospora lactis; Bact. aerogenes.*

Method. 1. Inoculate four tubes of dextrose broth with each organism.

2. Place one culture of each organism at each of the following temperatures: 5°, 25°, 37°, and 45°.

3. Note the growth as to vigor after twenty-four, forty-eight, seventy-two hours and seven days. Tabulate the data.

4. What is the natural habitat of each organism? Does this explain your results in any way?

What is the biological significance of the cardinal points of temperature? In what industries making use of microorganisms is the regulation of temperature especially important?

What inter-relations have the optimum, minimum and maximum temperature requirements of one species of microorganism?

What influence will the reaction of the medium have upon the extremes of temperature at which microorganisms will grow?

5. Discuss your results in detail and draw any conclusions permitted. Point out any practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 213–219, 263, 295–298, 390, 391, 457–470.

Lafar: Technical Mycology, Vol. I, pp. 75–77.

Jordan: General Bacteriology, Fifth Edition, pp. 78–80.

EXERCISE 22. THE EFFECT OF FREEZING UPON SPORE-FORMING AND NON-SPORE-FORMING BACTERIA

Apparatus. Small ice dish; thermometer; three tubes each of sterile cider, sterile milk, sterile broth and sterile wort; coarse salt; ice.

Cultures. *Sacch. cerevisiæ; Bact. lactis acidi; B. megaterium; Aspergillus niger.*

Method. **1.** Heavily inoculate the cider tubes with the yeast, the milk tubes with *Bact. lactis acidi*, the broth tubes with *B. megaterium* and the wort tubes with *Aspergillus niger.*

2. Incubate one set of cultures at 25° C.

3. Make sufficient freezing mixture of ice and salt to nearly fill the ice dish.

4. Carefully insert the two remaining sets of cultures in the freezing mixture and keep the freezing mixture at or below 0° C. for two hours.

5. Remove one set of tubes and incubate at 25° C.

6. Then place the ice dish containing the third set of cultures in the refrigerator (note the temperature).

7. Examine both sets of cultures at the end of twenty-four hours and forty-eight hours for growth.

8. Compare the three sets of cultures and note the variations from the normal type of growth. Tabulate your data.

9. Are all of these organisms pecilothermic? What are termed the cardinal points of temperature for microorganisms? What is the lowest temperature at which growth, even of the feeblest kind, is possible? What term is applied to organisms which grow best at low temperatures?

10. Give all results and answers in full and draw any conclusions permissible. Point out the practical applications that may be made.

REFERENCES

Fischer: Structure and Functions of Bacteria, pp. 73–75.

Marshall: Microbiology, Second Edition, pp. 214, 215, 218, 219, 261, 298, 390, 393, 470–471.

Lafar: Technical Mycology, Vol. I, pp. 75–77.

EXERCISE 23. TO DETERMINE THE THERMAL DEATH POINT OF MICROORGANISMS

Apparatus. Glass tubing with 2-3 mm. bore; file, three-cornered; fishtail burner; sterile filter paper; forceps; sterile broth or physiological salt solution for making suspension; thirty tubes sterile broth; small funnel, sterile.

Culture. *B. mycoides* * (students working side by side may use spores of a spore-former, and a non-spore former for comparison). *B. coli.*

Method. 1. Draw out glass tubing to capillary size by heating it over a fishtail burner.

* Successive cultures of *B. mycoides* must be *transferred daily* to fresh agar slants for several days previous to the time this experiment is to be done in order to eliminate the presence of spores. If the thermal death point of the spore is desired, cultures should be used as soon as abundant spore formation is evident.

2. Break the capillary tubes into lengths of 5–6 mm. and seal both ends in the flame.

3. Place thirty or more of the sealed capillary tubes in a large test tube plugged with cotton, and sterilize in the hot air sterilizer.

4. Using sterile broth or salt solution, make a suspension of the organism and filter into a sterile test tube to remove clumps; then transfer aseptically to a sterile Petri or Esmarch dish.

5. Immerse the tips of the capillary tubes in 1–1000 $HgCl_2$ for five minutes.

6. Rinse in sterile water and alcohol.

7. Lay the tip of the capillary tube on a sterile filter paper, sterilize a file in the flame and file off the end.*

8. Flame the tip of the capillary tube, and heat the sealed end slightly in the flame, handling with sterile forceps.

9. Immediately put the tip in the suspension of organisms. A quantity is drawn up by capillarity (surface tension) and contraction of the cooling air in the tube.

10. Seal the open end at once.

11. Place in the water bath at the proper temperature for the required time, according to the table.

Minutes	45°	50°	55°	60°	65°	70°	75°	80°	90°	100° C.
1										
5										
10										

This requires thirty capillary tubes for one organism.

12. Remove the capillary tubes and immerse in cold 1–1000 $HgCl_2$ five minutes.

13. Rinse in sterile water and alcohol.

* Some laboratory workers prefer to break off both ends of the capillary tubes. If this is done, care should be taken when sealing so as to sterlize them, not to heat the capillary tubes too long or too far back from each end.

14. Break the filled tip of the capillary tube with a flamed file, flame the tip and, holding over a tube of sterile broth, heat the empty end of the tube gently, so that expanding air will force out the suspension into the tube of broth. If the capillary tube is sterile on the outside, the whole thing may be dropped in the tube of broth after the end is broken off.

15. Mark the tubes *carefully* and incubate at 37°.

16. Record the results after twenty-four and forty-eight hours.

17. Does the period of incubation before the culture shows growth seem to bear any relation to the time exposed, or to the temperature to which the organism is exposed? Do spore-free cultures of spore-forming bacteria seem to have a greater resistance to heat than non-spore-forming organisms? Name two very resistant non-spore-forming organisms. Why are capillary tubes used instead of larger tubes?

18. Compare the results obtained by all students using spores for this experiment. Do spores of different species vary in their resistance to heat? In what industries is this knowledge of vital importance?

REFERENCES

Rosenau: Preventive Medicine and Hygiene, pp. 780–781.
Novy: Laboratory Work in Bacteriology, pp. 513–518.
Jordan: General Bacteriology, Third Edition, p. 217.
Marshall: Microbiology, Second Edition, pp. 220 and 221.

EXERCISE 24. TO DETERMINE THE RELATIVE EFFECT OF MOIST AND DRY HEAT ON BACTERIA

Apparatus. Ten tubes of nutrient broth (large tubes); ten sterile Esmarch dishes; ten sterile (flamed) cover-glasses; autoclav; steam sterilizer; hot-air sterilizer.

Cultures. Agar culture of a spore-forming organism (having spores at the time).

Milk culture of slimy milk organism, none-spore-forming.

Method. **1.** Make thick smears of each organism on five cover-glasses.

2. Place each cover-glass of the separate cultures in a sterile Esmarch dish and mark.

3. Place two Esmarch dishes of each culture in the hot-air sterilizer; heat to 120° C. and remove one dish of each culture after ten minutes at 120° C., the other two after thirty minutes.

4. Place two smears of each organism in the steam sterilizer; remove one of each after ten minutes, the two remaining after thirty minutes.

5. Place the two remaining Esmarch dishes in the autoclav and heat for ten minutes at 120° C.

6. When cool, transfer each of the cover-glasses to a tube of sterile broth; mark carefully.

7. Note in which tubes growth appears.

8. What is one of the most necessary factors for the prompt destruction of microorganisms by heat? Why?

Not considering moisture, what various conditions influence the destruction of microorganisms by heat? How are molds and yeasts influenced by moist and dry heat?

To what factors are the greater destructive powers of the autoclav due?

9. Give all data and results in full. Draw any conclusions possible and point out any practical applications.

REFERENCES

Hite, B. H., Giddings, N. J., and Weakley, Chas. E.: The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. Bul. 146, W. Va. Univ. Agr'l Expt. Sta., 1914.

Marshall: Microbiology, Second Edition, pp. 219–221, 464–466.

Lafar: Technical Mycology, Vol. I, pp. 101–105, Vol. II, Part I, p. 29.

Fischer: Structure and Functions of Bacteria, pp. 75–77.

EXERCISE 25. TO DETERMINE THE EFFECT OF PASTEURIZATION UPON THE GROWTH OF MICRO-ORGANISMS

Apparatus. 300 c.c. each of milk (not sterile) and of some fermenting fruit juice; water bath; two thermometers; four sterile 200 c.c. Erlenmeyer flasks; twenty-four tubes of dextrose agar; dilution flasks; twenty-four sterile Petri dishes; sterile 1 c.c. and 10 c.c. pipettes.

Method. **1.** Place 150 c.c. of milk in each of two 200 c.c. sterile Erlenmeyer flasks; do the same with the fruit juice.

2. Make three dilution plates each (1–100, 1–10,000, 1–1,000,000) from the milk and from the fruit juice in agar and incubate (inverted) at room temperature.

3. Place a flask of each nutrient liquid in the water bath (cold water) and heat rapidly to 75°–80° (thermometer in each flask), shaking the flasks frequently to obtain an even temperature throughout their contents.

4. Remove the flasks when the temperature reaches 80° C. and cool* them quickly.

5. Make dilution plates (1–10, 1–1,000, 1–10,000) in agar; mark each carefully. Place the flasks and plates at room temperature.

6. Place the other two flasks in the water bath (in cold water) and heat *slowly* up to 60° (thermometer in each flask). Keep at 60°–65° for twenty minutes.

7. Remove the flasks from water bath and cool* quickly.

8. Make dilution plates, using the same dilutions as before, and place the flask and plates at room temperature, marking each carefully.

9. Watch daily for signs of growth in each medium.

10. Make plates from each flask after six days, determining the range of dilutions by consulting your former

* It has been found by experiment that the *quick cooling* must take place through the temperatures 40°–36° C. in order to be most efficient in preventing further bacterial growth.

plates. Will the organisms have increased or decreased in this time? Why?

11. Compare the types of organisms on the plates before and just after pasteurizing and six days after pasteurizing. Examine each type microscopically. Of what does the predominant flora of each nutrient fluid consist before pasteurization? After pasteurization?

Note. The fruit juice may be saved for the experiment on metabiosis.

12. Count each set of plates and record the average number of microorganisms per c.c.

13. Plot the curve to show the destruction of microorganisms by pasteurization.

Compare the milk data with milk data and also the cider with cider.

14. Keep the original flasks for one or two weeks. If any marked changes occur, plate qualitatively and ascertain the type of organism causing the change.

15. How does the physical nature of the two nutrient substances influence their response to pasteurization? Give reasons for explanations offered.

What changes are brought about in milk by pasteurization? In cider or other fermenting fruit juice?

16. Give all data and results in full and draw any conclusions. Point out any practical applications that may be made.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 391–393, 461–464.

Russell and Hastings: Experimental Dairy Bacteriology, pp. 89–91.

Lafar: Technical Mycology, Vol. II, Part I, pp. 142–144.

EXERCISE 26. TO ILLUSTRATE THE EFFECT OF THE REACTION OF THE NUTRIENT MEDIUM UPON MICROORGANISMS

Apparatus. One liter of ordinary broth (should be enough for three students); normal NaOH; normal acid; four sterile 1 c.c. pipettes; 10 c.c. pipettes; sterile test tubes.

Cultures. *B. prodigiosus* (broth culture); *B. subtilis* (broth culture); *Oospora lactis* (broth culture); *Torula rosea* (broth culture).

Method. 1. By adding normal acid or alkali produce in 100 c.c. portions of ordinary broth the following reactions: −4.0, −3.0, −2.0, −1.0, 0, +1.0, +2.0, +3.0, +4.0, +5.0% normal, and titrate after re-adjusting the reaction, as a check.

2. Tube, using 9.9 c.c. in each tube (*mark the tubes plainly*), and sterilize (refiltration may be necessary before tubing in some cases).

3. Using a sterile 1 c.c. pipette, inoculate one set (ten tubes) with 0.1 c.c. of the broth culture of each of the above organisms (four sets) and incubate the tubes at room temperature.

4. Examine the tubes as often as possible for the first twenty-four to thirty-six hours, and record the tube or tubes in which macroscopic growth is first visible. What do you conclude as to the effect of the reaction of the medium in these instances?

5. Examine the tubes every day for seven days. Tabulate your observation. Note the range of reaction in which each organism is capable of growing. Does this range differ with different organisms? Explain the action occurring.

6. In each case inoculate heavily the first tube at either or both extremes, in which the organism fails to grow, from the tube just next it in series which shows growth. Does this freshly inoculated tube show signs of growth after

twenty-four to forty-eight hours? Explain the action which occurs.

7. Which organisms are acidophiles?

What is the optimum, the minimum and the maximum reaction for each organism according to this experiment?

What factors not considered in this experiment might influence results?

How would you determine the exact optimum reaction of an organism?

8. Give all data and results in full and draw conclusions. Point out any practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 235, 236, 298–300, 396, 442, 466, 550, 551.

Eyre: Bacteriological Technic, Second Edition, pp. 305, 306.

EXERCISE 27. TO DETERMINE THE INFLUENCE OF DIFFUSED LIGHT ON MOLDS

Apparatus. Sterile deep culture dish; tube of dextrose agar or gelatin; black paper.

Culture. *Rhizopus nigricans.*

Method. **1.** Pour a tube of agar into a deep culture dish.

2. When solid, inoculate with *Rhizopus nigricans.*

3. Wrap the dish closely in black paper so that no light can penetrate.

4. Cut a small narrow slit about 2 cm. long and 2 mm. wide in the top part of the paper and at the extreme edge and place the dish in a north window so that only diffused light will enter the aperture.

5. Allow the dish to stand ten days, then examine it. How does diffused light influence this mold?

6. What is the term applied to this type of action? How are mold spores influenced by light? What influence

does diffused light have on other microorganisms? Is it to be expected that other common molds, *Penicillium*, *Oospora*, *Aspergillus*, etc., would exhibit this same phenomenon?

7. Give all data and observations in detail. Draw any

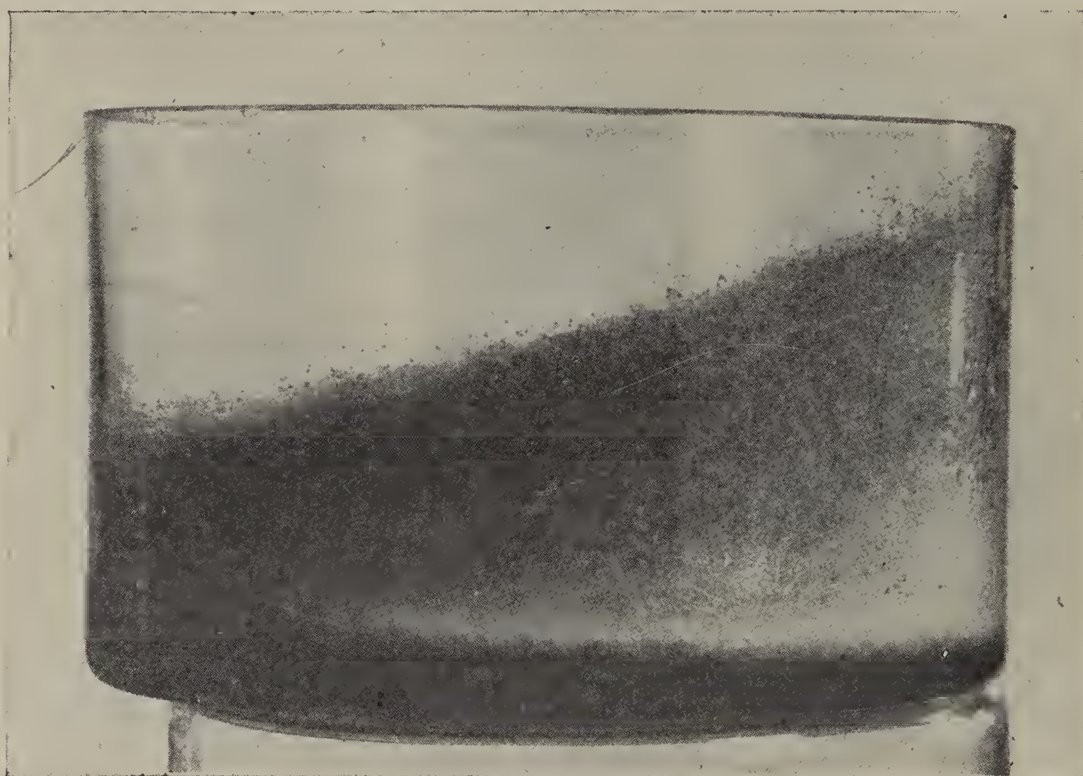


FIG. 44.—Phototropism Exhibited by *Rhizopus nigricans*. The mold was grown on gelatin with diffused light coming from the right side. (From Marshall.)

conclusions that follow and point out any practical applications.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 222–225, 263.
JORDAN: General Bacteriology, Fifth Edition, p. 81.

EXERCISE 28. TO SHOW THE INFLUENCE OF DIRECT SUNLIGHT UPON THE GROWTH OF MICROORGANISMS

Apparatus. Two tubes of sterile agar; two sterile Petri dishes; two sterile 1 c.c. pipettes; two tubes of sterile

Fig. 45.—Action of Direct Sunlight on Bacteria. These plates were heavily inoculated with *B. coli* and *B. prodigiosus*, respectively, and then were exposed bottom side up to the direct rays of the January sun for four hours. At the moment of exposure the figure 0, cut from black paper, was pasted to the plate, shading the bacteria underneath. After one, two and three hours the corresponding figures were pasted to the plates. The above picture was taken twenty-four hours after exposure, proving that three or four hours' exposure to direct sunlight weakens and may even kill bacteria. *B. prodigiosus* proved more sensitive than *B. coli*. (From Marshall.)

distilled water, salt solution or broth for dilution purposes; black paper; glue.

Cultures. *Ps. campestris; B. typhosus.*

Method. **1.** Inoculate a tube of sterile liquid heavily with *Ps. campestris*. Mix the contents well.

2. Place 1 c.c. of this suspension in a sterile Petri dish and pour the plate.

3. Duplicate with the *B. typhosus* culture, *placing the pipettes immediately after using* in 1–1,000 $HgCl_2$.

4. Cut any design out of black paper and paste on the bottom of the Petri dish.

5. Place the dish bottom side up in *direct* sunlight for two hours.

6. Set the dish away in the dark at room temperature. Observe the growth and explain. Which organism is the more sensitive to sunlight? Conclusions?

Note. *Heat B. typhosus plate* 1 *hour in steam before cleaning the Petri dish!*

7. What theories have been advanced as to the mechanism of destruction by direct sunlight? Does sunlight have any effect on bacterial spores?

How are other forms of organisms affected by light?

What is phototaxis? Do bacteria ever exhibit this phenomenon?

Which portion of the spectrum is most active?

What relation does the wave-length of light rays bear to the activity of the rays?

How do diffuse light, electric or other forms of artificial light, X-rays, radium rays, etc., compare with direct sunlight as to their action on bacteria in general?

8. Give all data and state results in full. Draw any conclusions that follow and point out any practical applications.

REFERENCES

Marshall, C. E.: Microbiology, Second Edition, pp. 222–225, 263.
Smith, Erwin F.: Bacteria in Relation to Plant Diseases, Vol. I, p. 71; Vol. II, p. 324.
Lafar: Technical Mycology, Vol. I, pp. 77–81.

EXERCISE 29. DETERMINATION OF THE PHENOL COEFFICIENT OF SOME COMMON DISINFECTANTS

(*Two students working together are required in this exercise.*)

Apparatus. Copper water bath; test-tube rack for above bath; forty-eight test tubes of uniform size containing exactly 5 c.c. of sterile nutrient broth (use a graduated burette or a similar apparatus for filling tubes); eight clean dry test tubes of *uniform* size; several (4 or 5) platinum loops, of 4 mm. *inner* diameter; sterile 1 c.c. pipettes with fine point; three clean 5 c.c. pipettes; phenol, 5%; mercuric chloride, or any unknown disinfectant 1 : 500; small funnel; filter paper to fit funnel; sterile test tubes; watch with second hand.

Culture. *B. typhosus,* twenty-four hour broth culture grown at 37° C.

Method. **1.** Place the filter paper in the funnel, wrap in paper and sterilize in the hot air.

2. Filter the twenty-four-hour broth culture of *B. typhosus* into the sterile test tube. This is for the purpose of removing clumps of bacteria and any foreign matter. Funnel and filter paper are to be treated immediately after use with 1 : 1000 $HgCl_2$.

3. Regulate the water bath at 20° C. and keep at this temperature.

4. Mark the thirty-two test tubes, each containing exactly 5 c.c. of nutrient broth, with the name of the disinfectant, the dilution, and the time exposed, according to the following table. Then place the tubes, in order, in the rack in the water bath.

5. Mark each set of clean, dry test tubes carefully with the name of the disinfectant and the dilution to be added (see table on p. 212), and place in each, 5 c.c. of the dilution of the disinfectant as indicated on the labels. Keep in a test-tube rack at 20° C. *Work with one disinfectant at a time.*

N. B. Have the assistant carefully keep track of the exact time of all operations, to the second.

In actual practice determinations are made oftener than every five minutes, two and one-half minutes being the standard interval. This requires the most careful attention of both operator and assistant.

6. Using the 1 c.c. pipette, add 0.1 c.c. of the culture to *one* tube of each dilution of the disinfectant and *mix quickly* with a sharp rotary motion of the tube.

7. At the end of one minute from the time of each separate operation, make a loop transfer from the tube of each dilution of the disinfectant inoculated with the culture into the corresponding tube of broth in the water bath.

Note. The assistant takes the tubes from the water bath and hands them to the operator, then, after the operation of transferring, returns the inoculated broth tube to the water bath, sterilizes the needle and places it in the most handy position for the operator.

8. This operation is then repeated; working as quickly as possible, add 0.1 c.c. of the culture to the remaining tubes of the different dilutions of the disinfectant.

9. When, *in each case*, the culture has been exposed for exactly five minutes, ten minutes and fifteen minutes respectively to the action of the disinfectant, a loop transfer is to be made to the corresponding tube of broth.

10. When *all* transfers are made, place the broth cultures at 37° C. Examine after forty-eight hours for growth and record growth as + or −.

Note. The *phenol coefficient of a disinfectant is the ratio of the strength of the unknown disinfectant which will kill a filtered 24 hr. broth culture of B. typhosus in a certain length of time, to the strength of phenol which will accomplish the destruction in the same length of time, the dilution of phenol taken as 1.*

For example: Suppose the weakest dilution of an unknown disinfectant required to kill *B. typhosus* in $2\frac{1}{2}$ minutes is 1 : 400 and the weakest dilution of phenol that kills *B. typhosus* in the same time is 1 : 100. And the weakest dilution of an unknown disinfectant required to kill

B. typhosus in 15 minutes is 1 : 600 and the weakest dilution of phenol required to kill *B. typhosus* in the same time is 1 : 200. Then according to the Hygienic Laboratory method the phenol coefficient would be

$$\frac{\frac{400}{100}+\frac{600}{200}}{2}=\frac{4+3}{2}=3.5.$$

This means that the unknown disinfectant is 3.5 times as effective for killing *B. typhosus* as phenol.

Disinfectants are often used in practice in the presence of organic matter. It is important to know to what extent organic matter affects the efficiency of a disinfectant. For detailed method see Hygienic Lab. Bul. No. 82, pp. 27–31.

11. Determine the approximate phenol coefficient of mercuric chloride according to the results of your experiment. How does this compare with results in literature?

METHOD OF MAKING DILUTIONS OF DISINFECTANT FOR TEST

1 part of 5% phenol + 1 part distilled water = 1 : 40 phenol.
1 part of 5% phenol + 4 parts distilled water = 1 : 100 phenol.
1 part of 5% phenol + 9 parts distilled water = 1 : 200 phenol.

1 part of 1 : 500 $HgCl_2$ + 1 part distilled water = 1 : 1000 $HgCl_2$.
1 part of 1 : 500 $HgCl_2$ + 3 parts distilled water = 1 : 2000 $HgCl_2$.
1 part of 1 : 500 $HgCl_2$ + 9 parts distilled water = 1 : 5000 $HgCl_2$.

METHOD OF RECORDING RESULTS

Disinfectant.	Time in minutes during which culture is exposed to action of disinfectant.					
	2½ min.	5 min.	7½ min.	10 min.	12½ min.	15 min.
Phenol 1 : 20.						
Phenol 1 : 40.						
Phenol 1 : 100. . . .						
Phenol 1 : 200. . . .						
$HgCl_2$ 1 : 500.						
$HgCl_2$ 1 : 1000. . . .						
$HgCl_2$ 1 : 2000. . . .						
$HgCl_2$ 1 : 5000. . . .						

12. What are some of the principal factors involved in the examination of disinfectants (pp. 12–20, Hyg. Lab. Bul. No. 82). How would each of these come into consideration in actual practice?

13. Give data and results in full. Draw any conclusions that properly follow and point out any practical applications.

REFERENCES

John F. Anderson and Thomas B. McClintic: I. Method of Standardizing Disinfectants with and without Organic Matter. Hygienic Laboratory Bul. No. 82 (1912), pp. 1–20, 34, 73.

S. Rideal and E. K. Rideal: Some Remarks on the Rideal–Walker Test and the Rideal-Walker Method. Jour. of Infectious Diseases, Vol. X (1912), pp. 251–257.

H. C. Hamilton and T. Ohno: Standardization of Disinfectants. Reprint No. 45 (1913), from Research Laboratory of Parke, Davis and Co., pp. 451–458.

—— The Bacteriological Standardization of Disinfectants. Reprint No. 65 (1914), from Research Laboratory of Parke, Davis and Co.

M. L. Holm and E. A. Gardner: Formaldehyde Disinfection with Special Reference to the Comparative Value of Some of the Proprietary Products. Journal of Infectious Diseases, Vol. VII (1910), pp. 642, 643, 650, 658–659.

Marshall: Microbiology, Second Edition, pp. 232–240.

Schneider, A.: Bacteriological Methods, Foods and Drugs (1915), pp. 230–263.

Report of the Committee on Standard Methods of Examining Disinfectants. Jour. of A. P. N. A., Vol. VIII, 1918, pp. 506–521.

Tanner, Bacteriology and Mycology of Foods, pp. 165–171.

EXERCISE 30. TO DETERMINE THE ACTION OF FORMALDEHYDE UPON THE MICROFLORA OF MILK

Apparatus. Fresh milk, skim or whole; 1% solution of formaldehyde; azolitmin solution; twelve sterile test tubes; H_2SO_4, concentrated *commercial.*

Method. 1. Make the following mixtures in sterile test tubes in *plain* milk, and *duplicate* in litmus milk (adding 2% azolitmin solution to the milk):

Milk.	Formaldehyde	Per cent Formaldehyde.
9.0 c.c. +	1 c.c. of 1%	= 0.1%
9.3 c.c. +	0.7 c.c. of 1%	= 0.07%
9.7 c.c. +	0.3 c.c. of 1%	= 0.03%
9.0 c.c. +	1 c.c. of 0.1%	= 0.01%
9.0 c.c. +	1 c.c. of 0.07%	= 0.007%
9.0 c.c. +	1 c.c. of 0.03%	= 0.003%

Place at room temperature.

2. Record the action in each tube, the time required for spoilage and the amount of formaldehyde necessary to preserve the milk.

3. What is the lowest per cent of formaldehyde that has *inhibitive* action? That has *preservative* action? What terms are applied to these different percentages in each case?

4. Make a "ring" test for formaldehyde as follows: Add several drops of concentrated commercial H_2SO_4 to each tube of *plain* milk, allowing it to run down the side of the tube as in making an ordinary "ring" test. A violet coloration at the junction of the H_2SO_4 with the milk demonstrates the presence of formaldehyde in the milk. The presence of ferric chloride, an impurity in commercial sulphuric acid, is essential to this test.

5. Did all percentages of formaldehyde used give this test? Did all percentages which *preserved* give the test?

Is formaldehyde a desirable preservative for milk? Why? Are any chemicals more desirable for this purpose than formaldehyde?

What are the main uses of formaldehyde? What is paraformaldehyde? Its use?

6. State your results in full and draw any conclusions that follow. What practical applications may be made?

REFERENCES

Marshall: Microbiology, Second Edition, pp. 238, 239 and 398.
Savage: Milk and Public Health, pp. 383–391.
Hawk: Practical Physiological Chemistry, Sixth Edition, p. 352.

EXERCISE 31. TO ILLUSTRATE SYMBIOSIS

Apparatus. Sterile 5 c.c. pipettes; three sterile 200 c.c. Erlenmeyer flasks; 450 c.c. skim milk; three tubes of litmus milk (sterile).

Cultures. *Bact. lactis acidi; Oospora lactis.*

Method. **1.** Place 150 c.c. milk in each flask and sterilize (Tyndall method).

2. Mark the flasks, *A*, *B* and *C*. Inoculate flask *A* with *Bact. lactis acidi,* flask *B* with *Bact. lactis acidi* and *Oospora lactis,* and flask *C* with *Oospora lactis* alone.

3. Make ten titrations, titrating every two or three days (not oftener) and record the titrations. Tabulate the data.

4. Plot curves. How do you explain the direction these curves take?

5. At the end of the titrations, make loop transfers from each flask into litmus milk tubes and watch these carefully in the next twenty-four to forty-eight hours. Record the results.

6. Does the action in the flasks appear to be symbiotic? If so, how is it shown?

Is this symbiosis desirable or not? Explain.

What other well-known examples of symbiosis occur in nature? Give a reason for your statement.

7. Give all data and results in full. Draw any conclusions that follow and point out any practical operations.

REFERENCES

MARSHALL: Microbiology, Second Ed., pp. 241, 242, 343–350 and 396.

NORTHRUP: The Influence of Certain Acid-destroying Yeasts upon Lactic Bacteria. Tech. Bul. No. 15, Mich. Expt. Sta., pp. 8–16, 32–34.

EXERCISE 32. TO ILLUSTRATE ONE OF THE PHASES OF MUTUAL RELATIONSHIP OF MICROORGANISMS

Apparatus. Sterile 5 c.c. pipette; 3 sterile 200 c.c. Erlenmeyer flasks; 450 c.c. sweet cider (that from pasteurization experiment may be used).

Cultures. *Sacch. ellipsoideus, Bact. aceti.*

Method. **1.** Place 150 c.c. of sweet cider in each flask.

2. Determine and record the reaction of the cider, then heat the flasks thirty minutes in the steam.

3. Cool the flasks and inoculate flask *A* with *Sacch. ellipsoideus.*

Fig. 46. Antibiosis. This peculiarity of growth is the result of the inhibitive action of metabolic products diffused through the medium. (Orig. Northrup.)

4. Determine the weight *at once* and then every day until the weight becomes constant.

5. Inoculate flask *B* with *Bact. aceti* and flask *C* with both *Bact. aceti* and *Sacch. ellipsoideus.*

6. Titrate *B* and *C* every two days. Titrate flask *A* only at the end of the experiment.

7. Determine the amount of alcohol formed in flask *A* by distilling the contents of the flask and determining the specific gravity of the distillate.

How much CO_2 was given off? Calculating from this amount, how many grams of sugar ($C_6H_{12}O_6$) were present in the flask? What percent sugar was this solution?

What was the theoretical amount of alcohol present?

8. Plot curves showing the acid formation in each case. Explain the direction which these curves take.

9. Explain the mutual action and the changes which occur.

10. What enzymes are responsible for each change? Write out the chemical equations for each change, giving enzyme concerned in each case.

Was the theoretical amount of alcohol changed into acetic acid? Give a reason for what really does happen.

What phase of mutual relationship is illustrated?

What is the classical example of this type of mutual relationship?

11. Give data and observations in full and draw conclusions. Point out any practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 242, 243, 329, 538–550.

Lafar: Technical Mycology, Vol. I, pp. 394–397; Vol. II, Part 2, pp. 473–481, 511–515.

EXERCISE 33. TO DEMONSTRATE THE EFFECT OF THE METABOLIC PRODUCTS OF BACT. LACTIS ACIDI ON ITS ACTIVITIES

Apparatus. Two sterile 200 c.c. flasks. 200 c.c. sweet skim milk; azolitmin solution; apparatus for titration; sterile dilution flasks; sterile Petri dishes; sterile 1 c.c. pipettes; sterile 5 c.c. pipettes; sterile 10 c.c. pipettes; ten to fifteen tubes of sterile litmus milk.

Culture. *Bact. lactis acidi* (twenty-four-hour culture).

(*At least two weeks should be allowed for the completion of this experiment.*)

Method. **1.** Place 100 c.c. skim milk, +1.0 to +1.5% normal (record acidity before adding azolitmin), in each flask and sterilize by the autoclav.

2. Mark the flasks *A* and *B*.

3. Inoculate each flask with a loopful of a twenty-four-hour milk culture of *Bact. lactis acidi*. Mix well with a needle and plate dilutions 1–10, 1–100, 1–10,000 from flask *A* for obtaining the initial number of *Bact. lactis acidi* introduced per c.c.

4. Continue as follows:

Flask A:
- *2d day,* titrate and use dilutions 1–10,000, 1–100,000 and 1–1,000,000.
- *3d day,* titrate and use dilutions 1–1,000,000, 1–10,000,000 and 1–100,000,000.
- *5th day,* titrate and use dilutions 1–100,000, 1–1,000,000 and 1–10,000,000.
- *7th day,* titrate and use dilutions 1–1,000, 1–10,000 and 1–100,000.

N. B. *Shake the flask of milk well each time before titrating and making dilutions.*

5. Titrate and plate every third day thereafter, until the acidity remains constant.

6. In flask *B* from day to day note in millimeters the extent of the re-oxidation of the azolitmin.

7. *Flask B.* Without disturbing the milk any more than necessary, make a loop transfer every day or so for 10 to 14 days from this flask into a tube of sterile litmus milk.

What occurs in each case? In what respects does flask *B* check up with flask *A?* Give explanations for similarity or dissimilarity of actions occurring.

8. Milk contains on an average about 4.5% lactose.

Has this sugar been fermented entirely to lactic acid? Explain what really occurs.

9. What titre would milk containing 5% lactose have if this sugar were entirely changed to lactic acid?

Does any lactic-acid-producing organism approximate this reaction (in milk) at the height of its activity?

10. Give reasons for what occurs in each flask. What practical applications may this experiment have?

11. Tabulate your results and plot number and acidity curves. Explain these curves.

12. Draw any conclusions that follow from the above and point out any practical applications.

REFERENCES

RAHN, O.: The Fermenting Capacity of a Single Cell of *Baci. lactis acidi*. Tech. Bul. No. 10, Mich. Exp. Sta., p. 25, *et al*.

MARSHALL: Microbiology, Second Edition, pp. 217, 383–385.

PART III

APPLIED MICROBIOLOGY

AIR MICROBIOLOGY

EXERCISE 1. QUANTITATIVE BACTERIAL ANALYSIS OF AIR

Apparatus. One carbon tube, dia. 15 mm.; cork stopper, perforated, to fit carbon tube; short piece of glass tubing bent at right angles; sand which has passed through a 150 mesh sieve; 8-liter aspirator bottle complete with rubber stoppers and glass tubing; sterile test tubes * containing 10 c.c. of sterile physiological salt solution; sterile 1 c.c. pipettes; four sterile Petri dishes; four tubes of sterile agar for plating; sterile agar slants; tubes of sterile broth; tubes of sterile litmus milk.

Method. **1.** Prepare a sand filter aeroscope by placing a layer of cotton in the bottom of the carbon tube.

2. Upon this place 1 cm. of sand which has been run through a 150 mesh sieve.

3. Insert a cork stopper through which is passed a bent glass tube plugged at the outer end with cotton.

4. Sterilize the apparatus in hot air oven.

5. Place 8 liters of water in the aspirator bottle and mark the level of this amount of liquid.

6. Adjust the delivery tube so that it aspirates one liter of air per minute.

* For convenience in shaking the sample, it is recommended to use test tubes with aluminum screw caps, having cork packing.

7. Attach the aeroscope (lower end of carbon tube) to the aspirator so that the aspirated air will be filtered through the sand.

8. Remove the cotton plug from the upper end of the aeroscope and filter 8 liters of air in approximately eight minutes.

9. Using "aseptic" precautions, transfer as much sand as possible to one of the tubes of sterile salt solution.

10. Mix well by bumping the tube against the hand at least fifty times (do not wet the cotton plug).

11. Then, with a sterile 1 c.c. pipette, transfer 1 c.c. of the suspension to each of four Petri dishes and pour plates.

12. Incubate two plates at 37° C. for two days, and the remaining two plates at room temperature for five days.

13. Count at the end of these respective periods and determine the number of bacteria per liter. How do your counts compare with air counts obtained by other students? from other data? (See Marshall's Microbiology, p. 251.)

Make separate counts of molds and identify them as far as possible.

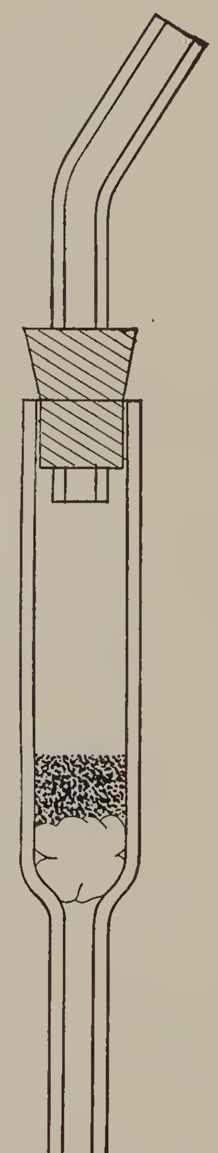


Fig. 47.—Modified Standard Aeroscope. (Ruehle and Kulp.)

14. Make sub-cultures of different types on agar and study their cultural characteristics on this medium.

15. Transfer these cultures to tubes of broth and litmus milk and note their action on these media. Draw conclusions from these results.

16. What morphological types are found? Are any of the types of bacteria present constantly found in air? What are the sources of microorganisms in the air?

Are any of the types isolated related to pathogenic forms? May pathogenic bacteria be isolated from air? If so, under what circumstances?

How do microorganisms enter the air? What types of microorganisms are most apt to be present in air? What is the explanation for this?

What other methods may be employed for obtaining quantitatively the bacteria in the air?

Of what importance is the quantitative or qualitative determination of microorganisms in air?

17. Give data and results in full and draw any conclusions permitted. Point out any practical applications of the above.

REFERENCES

Ruehle, G. L. A. and Kulp, W. L.: Germ content of stable air and its effect upon the germ content of milk, Bul. 409, N. Y. Expt. Sta., 1915.

Marshall: Microbiology, Second Edition, pp. 247–253.

Eyre: Bacteriological Technic: 2d Ed. (1913), pp. 468–470.

Besson: Practical Bacteriology, Microbiology and Serum Therapy, transl. by Hutchens (1913), pp. 862–867.

Chapin, C. V.: The air as a vehicle of infection. Jour. Amer. Med. Ass'n., Vol. LXII, pp. 423–430 (1914).

Winslow, C. E. A.: Bacteriology of air and its sanitary significance. Cent. f. Bakt. Abt. II. Bd., 42, p. 71 (1914).

Winslow, C. E. A. and Brown, W. W.: The microbic content of indoor and outdoor air. Mo. Weather Rev., Vol. XLII (1914), pp. 452–453. Abst. in Exp't. Sta. Record, Vol. XXXII, No. 3 (1915), p. 211.

WATER AND SEWAGE MICROBIOLOGY

EXERCISE 1. TO DEMONSTRATE THE REACTIONS OF DIFFERENT CULTURE MEDIA TO COLON-TYPHOID ORGANISMS

Apparatus. Glass rod bent at a right angle; five plates Endo's medium; five plates litmus lactose agar; five eosin-methylen blue medium; five triple sugar agar slants; five fermentation tubes of lactose broth; five fermentation tubes of saccharose broth; five fermentation tubes of adonite broth; five fermentation tubes dextrose potassium phosphate broth; five tubes tryptophane broth; five fermentation tubes each of maltose, mannite, dextrose broth; five gelatin plates; agglutinating serum for each organism.

Culture. *B. coli communis*; *B. coli communior*; *B. aerogenes*; *B. cloacae*; *B. typhosus*.

Method. 1. Make dilute suspension in sterile water of each organism. Inoculate one plate each of Endo's medium, litmus lactose agar, and eosin-methylen blue medium as follows:

2. Pour the plates and allow them to harden. Then place in the incubator, with covers removed, for fifteen minutes, or tilt the covers slightly and expose in the laboratory to dry the surface. Dip the tip of a glass rod bent at a right angle into the suspension of the organism. Touch the tip of the glass rod containing a drop of suspension to the center of the plate. With the heel of the rod, draw out the drop to the edge of the plate. Divide the plate into four sectors with a wax pencil, and inoculate each sector by drawing the glass rod around the plate, lifting the rod between each sector. This will give four distinct sectors, each with a different dilution. The best isolated colonies usually appear in the last sector.

3. Incubate the plates at 37° C. for twenty-four hours and note the typical growths on each medium.

4. Fish typical colonies of each of the various organisms onto triple sugar medium. Incubate at 37° C. for twenty-four hours. Record the results.

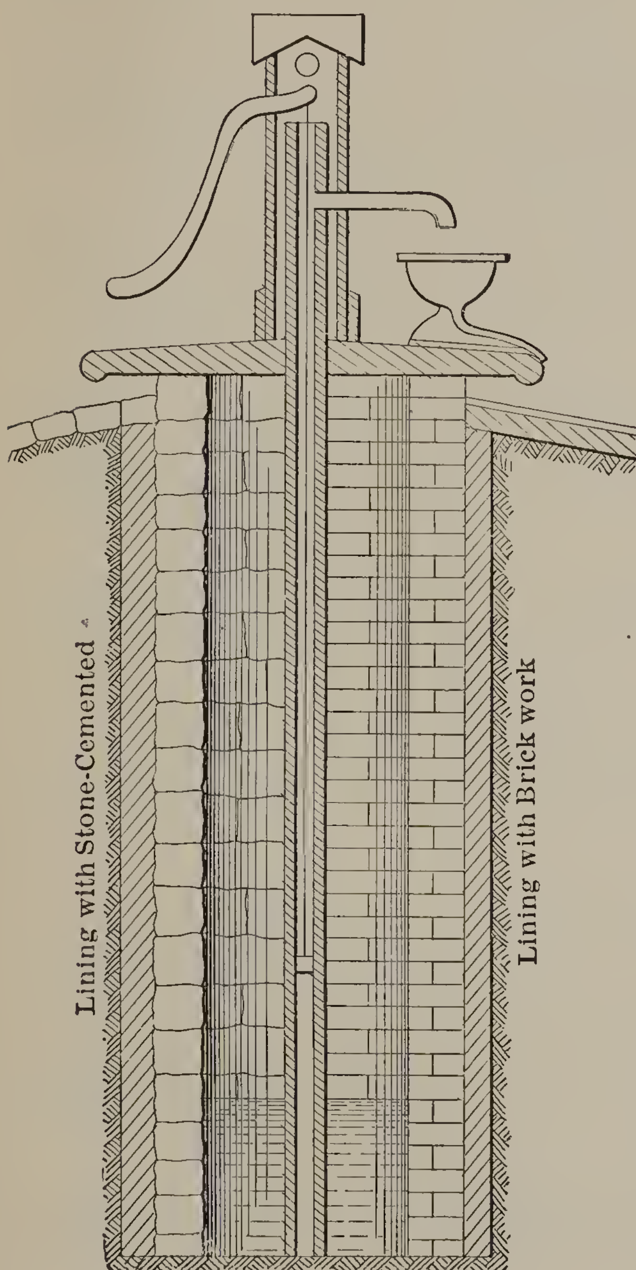


FIG. 48.

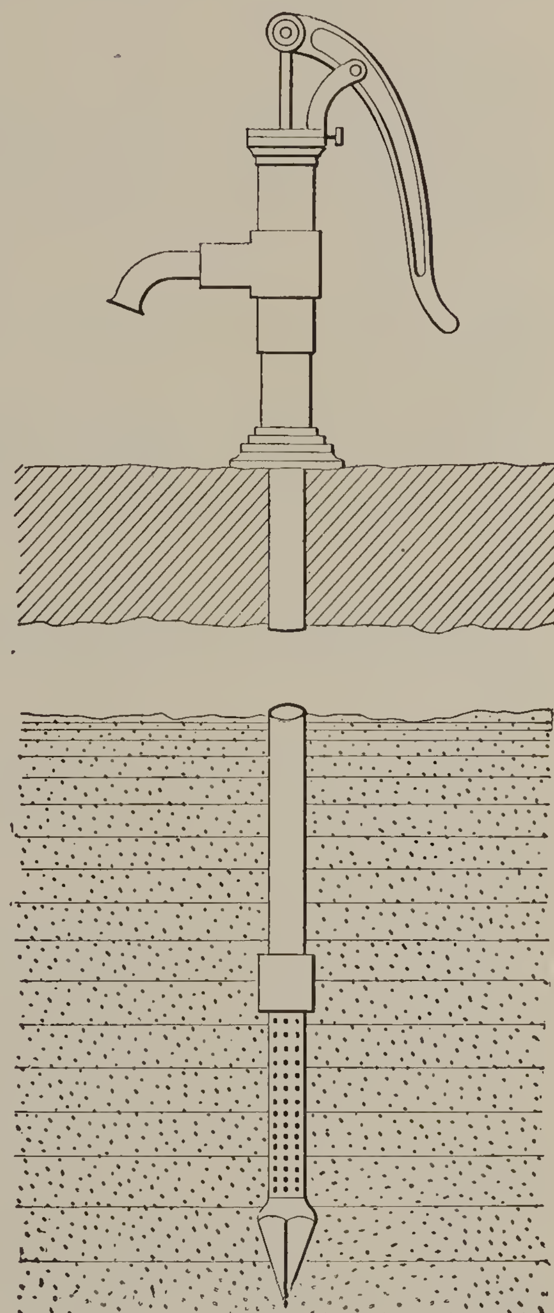


FIG. 49.

FIG. 48.—A Model Dug Well Constructed to Avoid Microbial Contamination of Water. (From Gerhard's Sanitation, Water Supply and Sewage Disposal of Country Houses.)
FIG. 49.—A Shallow Driven or Tube Well. (From Gerhard.)

5. If agglutinating sera is available, run agglutination tests for each organism. (For details of test see Exercise 8, page 314.)

6. Inoculate each organism into the following fermentation tubes: lactose, saccharose, adonite, maltose, mannite, and dextrose broth.

7. Incubate at 37° C. for forty-eight hours, noting gas-formation at the end of twenty-four and forty-eight hours.

8. Inoculate each culture into dextrose potassium phosphate broth and incubate at 30° C. for five days.

9. Note the gas-formation at the end of twenty-four and forty-eight hours. Allow to incubate for five days; at the end of this time apply the methyl red test and the Voges-Proskauer test.

10. Inoculate each culture into tryptophane broth. Incubate forty-eight hours at 37° C. Test for indol production.

11. Make a gelatin plate from each culture and incubate at 20° C. for seven days. Note the growth at two-day intervals.

12. Describe the typical growths on Endo's medium, litmus lactose agar, triple sugar slants, and eosin-methylen blue medium.

Describe characteristic growth on each medium.

Are the growths sufficiently characteristic to enable you to fish colonies with any degree of certainty?

13. Of what value are the different sugar fermentations? What are the advantages of triple sugar? Why is gelatin used?

14. What use is made of the methyl red, Vosges-Proskauer and indol tests?

REFERENCES

Standard Methods of Water Analysis, American Public Health Association (1913), pp. 77–80, 88–96, *et al.*

Prescott and Winslow: Elements of Water Bacteriology, Third Edition, pp. 1–51, 215–228.

Marshall: Microbiology, pp. 192–204.

Savage: Bacteriological Examination of Water Supplies (1906), pp. 192–193, 194–264.

THRESH: Examination of Waters and Water Supplies, Second Edition, (1913), pp. 184–271, 488–524.
DON and CHISHOLM: Modern Methods of Water Purification (1911), pp. 260–271.

EXERCISE 2. A COMPARISON OF THE TREASURY AND STANDARD METHODS OF BACTERIOLOGICAL WATER ANALYSIS.

Apparatus. Sterile 500 c.c. flask for collecting water samples; 25 c.c. of Endo's medium or 25 c.c. of litmus lactose agar; eight agar slants; eight tubes plain agar; four tubes gelatin; six improved Durham's fermentation tubes; eight Smith fermentation tubes.

Cultures. Water from the local water system should be used for the experiment. This method can be used also for water from deep wells, springs, etc.

Method. *Collection of samples.*

1. Flush the water pipes thoroughly by allowing the water to run, or by pumping, at least thirty minutes.

2. Hold the collection flask, mouth downwards, remove the plug and still holding in this inverted position, wash the mouth off with the running water, then fill quickly and replace the plug. *The plug must not be laid down during this process.*

3. The sample must be analyzed *at once.* In routine work, if this is not practicable, place the sample on ice and analyze *as soon as possible.* Samples kept at 10° C. or less should never be left over a maximum of six hours before analysis.

Method A. *For analysis.* (Adapted from Treasury method.)

1. Plate immediately in duplicate, 1 c.c., 0.5 c.c. and 0.1 c.c. of the sample direct on standard agar.

2. Incubate plates for twenty-four hours at 37° C. and count at the end of this time.

3. Inoculate five fermentation tubes, containing 30 cc. of lactose peptone broth, with 10 c.c. portions of the sample of water.

4. Incubate these tubes for forty-eight hours at 37° C. Note gas formation at end of twenty-four and forty-eight hours.

5. From each tube showing gas greater than 5% of the closed arm of the fermentation tube, make two plates using Endo's medium or litmus lactose agar. (As in Exercise 1, step 2).

6. Incubate forty-eight hours at 37° C.

7. Fish one or more well-isolated characteristic colonies from the plate and plant into a lactose-broth fermentation tube.

8. Incubate for forty-eight hours at 37° C. and note gas production. Record results at end of twenty-four and forty-eight hours.

Method B. (Adapted from Standard methods.)

1. Plate 1 c.c., 0.1 c.c. of water sample in duplicate on gelatin and plain agar. Incubate gelatin at 20° C. and agar at 37° C. for forty-eight hours. Count colonies and record results at end of twenty-four and forth-eight hours.

2. Inoculate fermentation tubes with 10 c.c., 1 c.c., 0.1 c.c. portions of sample to be tested.

3. Incubate at 37° C. for forty-eight hours. Examine each tube at the end of twenty-four and forty-eight hours and record gas-formation as follows:

(*a*) Absence of gas-formation.

(*b*) Gas-formation occupying less than 10% of the closed arm.

(*c*) Gas-formation occupying more than 10% of the closed arm.

4. If at the end of twenty-four hours no gas is present or gas-formation less than 10%, incubation should continue to forty-eight hours.

5. Smear one drop of broth from fermentation tube

showing gas-formation, from the smallest amount of water tested, using Endo's medium or litmus lactose agar. (See step 2, Exercise 1.)

6. Incubate the plates at 37° C., for eighteen to twenty-four hours.

7. Fish two or more typical colon-like red colonies, transferring each to an agar slant and a lactose broth fermentation tube.

8. Incubate agar slants and fermentation tubes at 37° C. for forty-eight hours. Note gas formation in fermentation tubes and make a microscopic examination of agar slants for non-spore-bacilli.

9. If no typical colonies appear upon plates made in step 5 at the end of twenty-four hours, incubate the plates for another twenty-four hours.

10. At the end of this time transfer two colonies most likely to be *B. coli* to agar slants and lactose broth fermentation tubes.

11. Incubate at 37° C. for forty-eight hours. Note gas-formation and make a microscopic examination of agar slants for non-spore-forming bacilli.

12. Which method is more accurate, simple and convenient? Compare the value of the two methods in routine water analysis as far as your experience permits.

13. Point out the advantages and disadvantages of each method.

14. Record and compare the number of types of organisms developing on the agar and gelatin plates. Explain why results vary on different media.

15. What other methods are used to determine the potability of water? Discuss these.

Why is an incubation period of forty-eight hours used?

Why isn't the fermentation of lactose sufficient evidence of *B. coli*?

REFERENCES

Standard Methods of Water Analysis, 1913 edition, pp. 79–82, 87–88, 92, 95–102.
Prescott and Winslow: Water Bacteriology, Third Edition, pp. 61–201, 228–265.
Savage: Bacteriological Examination of Water Supplies, pp. 27–69.
Marshall: Microbiology, pp. 97, 108, 162, 182, 204, 212, 221, 323–324.

EXERCISE 3. BACTERIOLOGICAL ANALYSIS OF SEWAGE, SEWAGE EFFLUENTS, ETC.

Apparatus. Sterile 500 c.c. flask; 99 c.c. dilution flasks; 90 c.c. dilution flasks; six triple sugar slants; three tubes litmus lactose agar or three tubes Endo's medium; three lactose broth fermentation tubes; ten tubes plain agar; ten tubes gelatin; three tubes eosin-methylene blue medium.

Cultures. Material for this may be obtained from sewer, septic tank, sewage effluents of septic tanks or city sewer system emptying into lake, river, etc., or from any water supply suspected of gross sewage contamination.

Method. **1.** Collect the sample in a sterile 500 c.c. flask, using same precautions as in exercise 1.

2. This sample must be analyzed *at once.*

3. Plate in duplicate on standard agar and gelatin using dilutions of 1 : 100, 1 : 1000, 1 : 10,000, 1 : 100,000, 1 : 1,000,000.

4. Incubate agar and gelatin plates at 37° C. and 20° C. respectively for forty-eight hours and count. Count gelatin every two days until end of seven days and estimate ratio of non-liquefying to liquifying colonies.

5. Inoculate 1 c.c., 0.1 c.c., 0.01 c.c. of sample into lactose broth fermentation tubes.

6. Incubate until gas (at least 10%) is formed not to exceed forty-eight hours.

7. Smear one drop from tubes showing gas on Endo's medium or litmus lactose agar, using a glass rod bent at a right angle. (See Exercise 1, step 2.)

8. Fish two or more typical colon colonies on triple sugar agar slants.

9. Plate 1 c.c. from the 1 : 1000, 1 : 100,000 and 1 : 1,000,000 dilution, using eosin-methylene blue medium.

10. Incubate twenty-four to forty-eight hours at 37° C.

11. Fish two or more typical colonies from each medium onto triple sugar slants. Incubate at 37° C. for twenty-four hours and record growths.

12. Examine some of the material under a hanging drop for protozoa and note different types present.

13. What is the significance in sewage of a large number of liquefying organisms? Compare the number of colonies developing on gelatin with the number on agar.

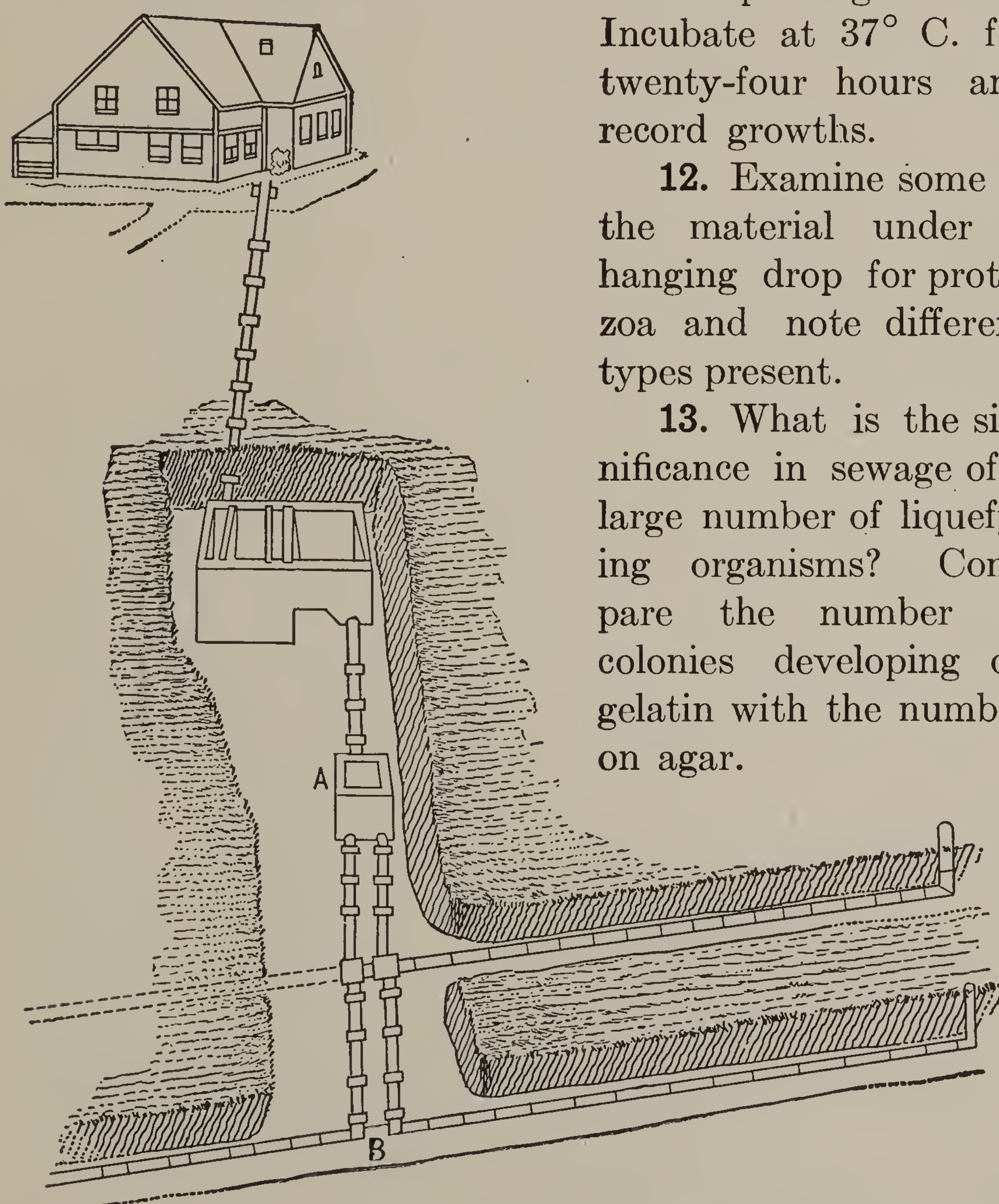


Fig. 50.—General Ground Plan of Individual House Sewer System Showing Exit from House, Anaerobic Tank, (A) Switch, and (B) Subsurface Irrigation Tile.

14. Discuss the relative merits of eosin-methylene blue medium, Endo's medium, and litmus lactose agar in identifying colon-typhoid organisms.

15. What is the significance of protozoa.

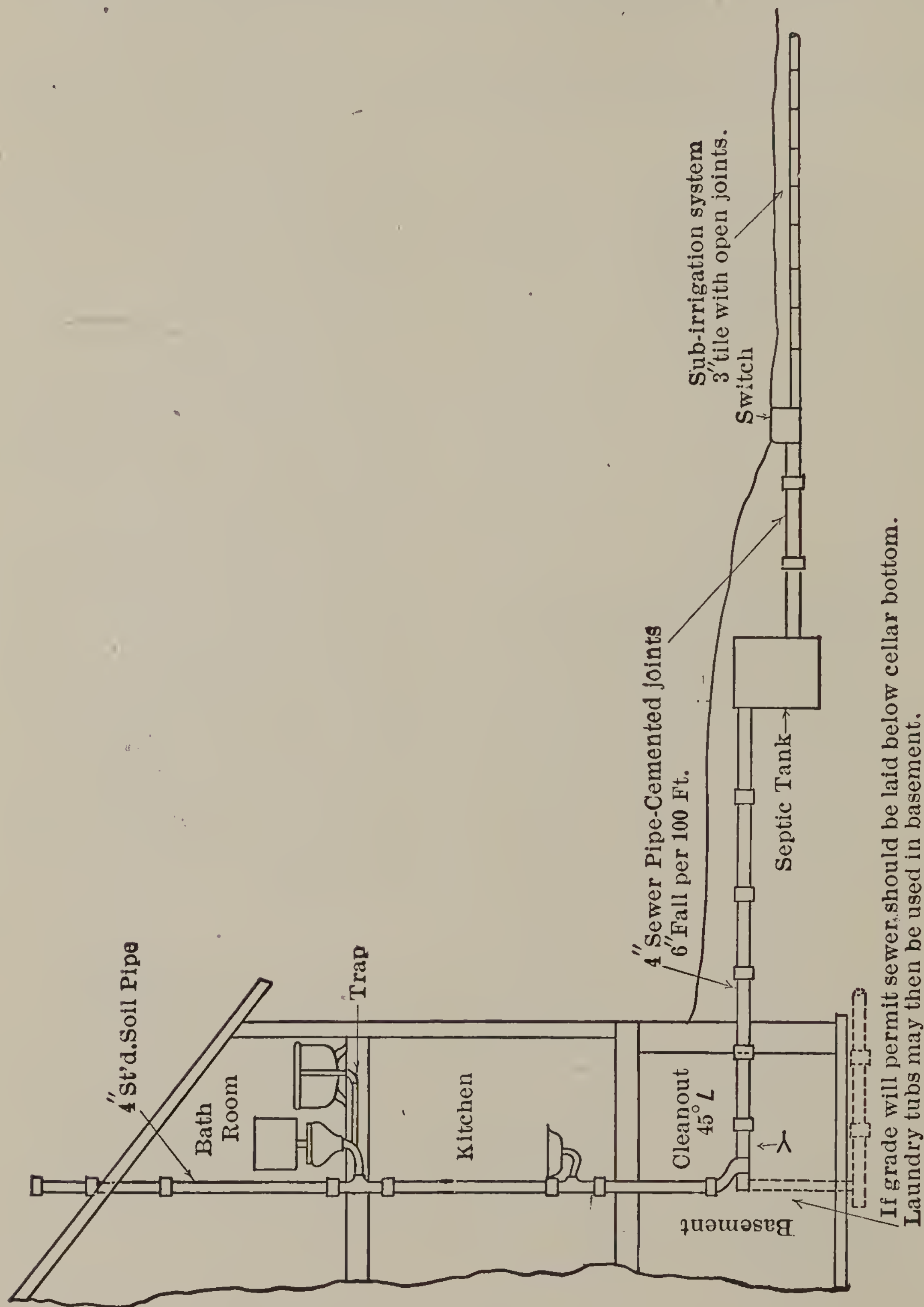


FIG. 51.—Sectional View of Same Sewer System.

REFERENCES

TANNER, F. W.: Bacteriology and Mycology of Foods, pp. 339-356.

PRESCOTT, S. C., and WINSLOW, C.-E. A.: Elements of Water Bacteriology. Third Edition, pp. 228–243.

Kinnicutt, L. P., Winslow, C.-E. A., and Pratt, R. W.: Sewage Disposal, pp. 1–20.

Whipple, G. C.: The Microscopy of Drinking Water, 3d Ed., pp. 8–13.

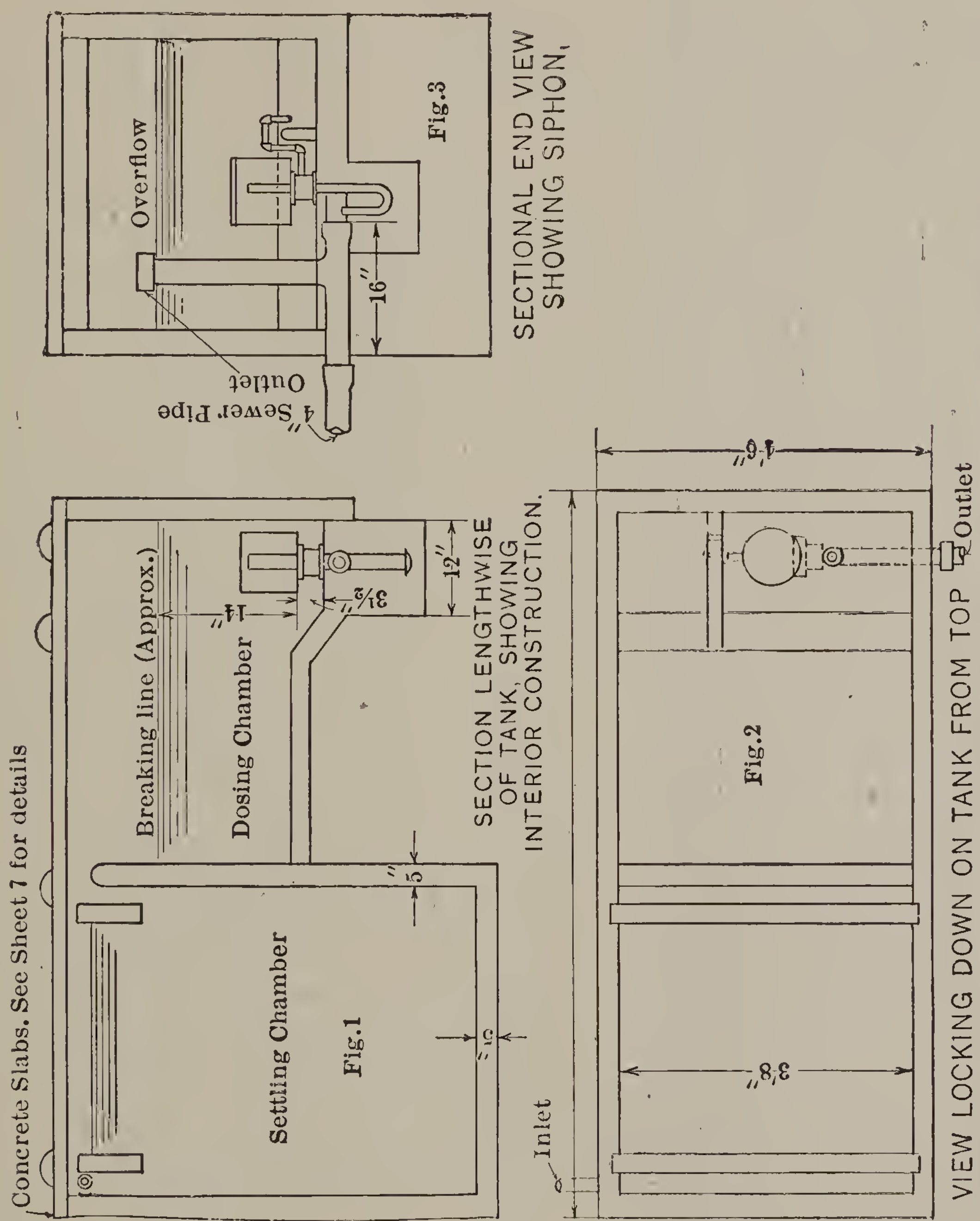


Fig. 52.—Plan of Anaerobic Tank Showing Longitudinal Section, Surface View and End View.

EXERCISE 4. A STUDY OF MODERN METHODS OF WATER PURIFICATION

Apparatus. A 3-foot glass tube, 3 cm. diameter; alum; can of fresh chloride of lime; 23 tubes litmus lactose agar; 23 tubes gelatin; 46 sterile Petri dishes; 20 sterile dilution flasks; various grades of sand and gravel, mortar.

Culture. Sewage effluent, river water or water suspected of gross polution.

Method A. **1.** Draw out one end of a 3-foot glass tube

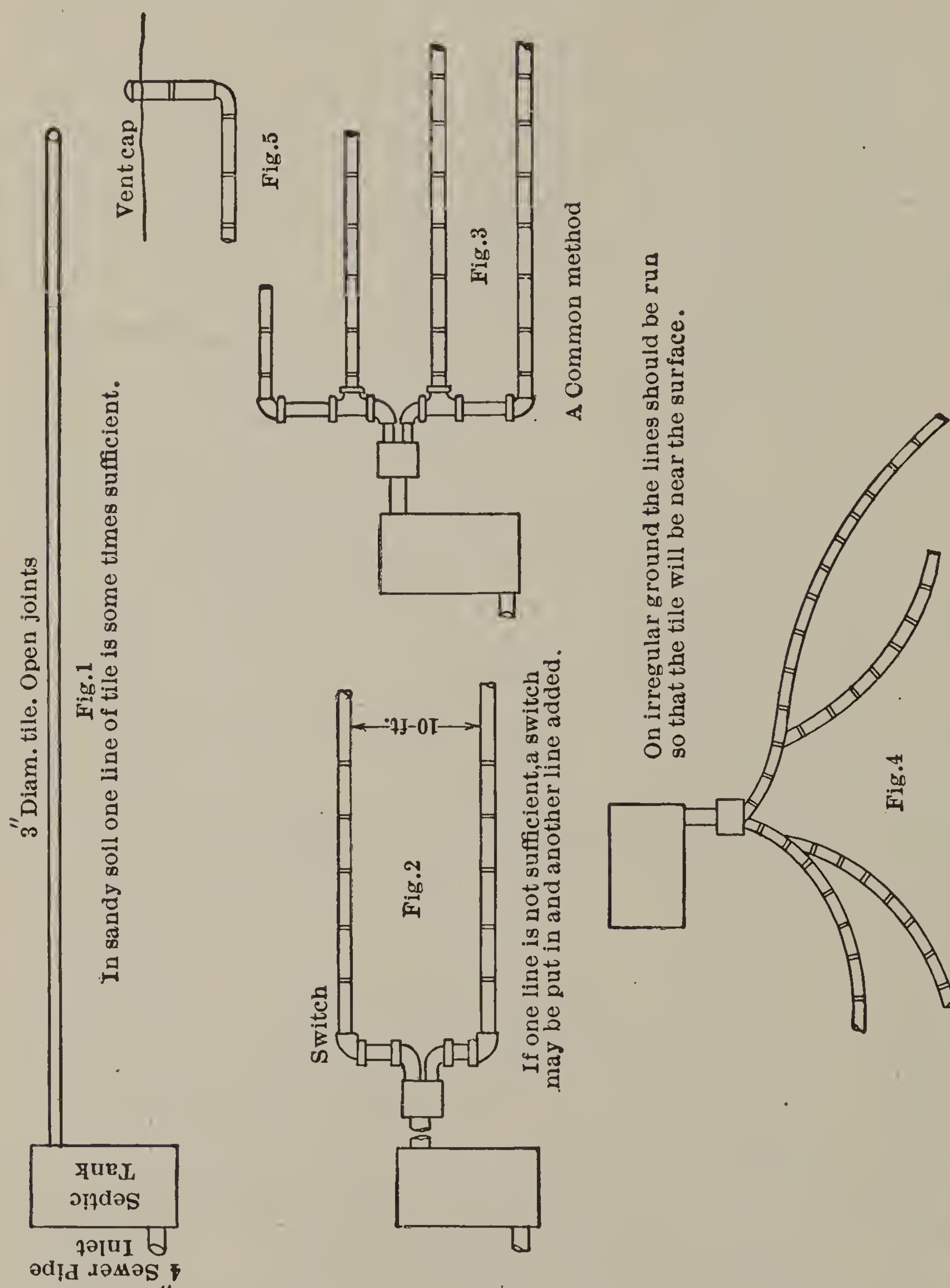


Fig. 53.—Details of Sub-surface Disposition of Effluent from Anaerobic Tank. (Figs. 50–53 from material for public distribution from Farm Mechanics Department, Mich. Ag. College, O. E. Robey.)

having a diameter of at least 3 cm. to a small opening (see Fig. 51).

2. Place gravel and sand in the tube, building it up as follows:

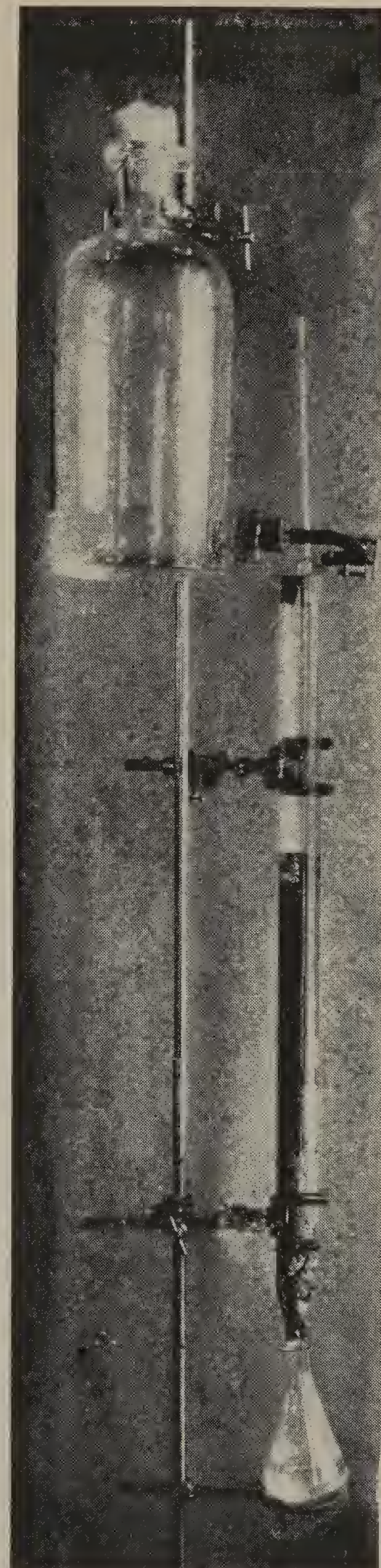


Fig. 53a.—Filter Used in Ex. 4.

1 to 4 inches of coarse gravel ($\frac{3}{8}$ to $\frac{3}{4}$ inch in diameter.)

4 to 8 inches of coarse sand.

8 to 12 inches of fine sand.

3. Wash and grade material by forcing water through the filter from the bottom.

4. Drain, plug both ends with cotton and sterilize in autoclav.

5. Set up apparatus following the diagram (Fig. 53a).

6. Build up scum of $Al_2(OH)_3$ on the surface of the filter bed by using alum water having a low bacterial content. (0.05 to 0.1 gm. alum per liter of water.)

7. Plate sample to be treated, using suitable dilutions (see Ex. 2) on litmus lactose agar and gelatin.

8. Treat with alum (using same amount as in step 6) and filter remaining portion of sample.

9. Plate on litmus lactose agar and gelatin at least four samples collected from filter at intervals of 15 minutes using suitable dilutions.

10. Count agar plates at the end of 48 hours and gelatin plates at the end of 2 days and 5 days. Record results.

Method B. 1. Determine the amount of available chlorine in the chloride of lime to be used by the following procedure:

(a) Remove the cover from the can of fresh chloride of lime and

obtain a vertical section by means of a sampling tube, forcing it to the bottom of the can by a rotary motion. Remove sample from the tube and mix thoroughly.

(*b*) Weigh out 5 gms. and grind in a mortar with 50–70 c.c. of water; wash into a 250 c.c. volumetric flask and make up to 250 c.c. Shake well.

(*c*) Allow to settle, then remove 10 c.c. by means of a pipette and titrate by the following method:

Bunsen's method: Add 10 c.c. of a 5% solution of potassium iodide and 0.5 c.c. glacial acetic acid and titrate with sodium thiosulphate (24.8 gms. of the C. P. crystalline salt and 1 c.c. of chloroform per liter) using starch solution as an indicator. Each c.c. of thiosulphate solution used equals 1.755 per cent. of available chlorin.

2. Prepare a solution of the chloride of lime containing 2% available chlorine. Mix well and allow to stand overnight before proceeding with the experiment.

3. Treat the filtered and unfiltered water with the chloride of lime solution, using 0.16 and 0.64 part of chlorine to one million parts of water. (See Hooker, "Chloride of Lime in Sanitation," p. 11.)

4. Shake vigorously for 1 minute and allow to stand 1 hour.

5. Plate on litmus lactose agar and gelatin using 1 c.c. and 0.1 c.c. direct and dilutions of 1–100 and 1–10,000.

6. Count agar plate at end of 48 hours; Count gelatin plates at end of 2 and 5 days, and record results.

7. What effect has filtration on chlorination? What is the comparative efficiency of filtration and chlorination in the case of raw water?

8. What part does alum play in filtration? What is the chemical action of chlorinated lime? Explain the reason of the arrangement of the various layers in the filter.

REFERENCES

Don and Chisholm: Modern Methods of Water Purification, pp. 59–153.
Hooker, A. E.: Chloride of Lime in Sanitation, pp. 1–34.
Race, Joseph: Chlorination of Water, p. 14–49, 79, 132–147.

BACTERIOLOGICAL WATER ANALYSIS

Sample No. . . . . Date. . . . . 19. . .
Name of sender. . . . .
Address. . . . .
Source of water. . . . .
Surroundings. . . . .
Temperature. . . . .
Appearance. . . . .
Odor . . . .
Remarks. . . . .

WATER QUESTIONNAIRE

"A report upon any given water sample is a scientific deduction, based upon local conditions and environments as well as upon the laboratory findings, and all available information should invariably accompany the sample to be examined." (Michigan Department of Health.)

NOTE.—*This sheet must be filled out as completely as possible and returned to us before we can send you a report since this information is necessary in making a decision as to the quality of the sample.*

Name. Date of shipping. . . . . 19. . . . M.
Address. Date received. . . . . 19. . . . M.
Source of sample:
Lake or river. . . . . Does sewage or other wastes empty into water. Describe fully. . . . .
. . . .
Kind of well—shallow (open or driven) drilled. . . . .
Depth. . . . .
Soil. . . . .
Subsoil. . . . .
Bed rock. . . . . Depth. . . . .
Are walls and top water-tight?. . . . .
Distance of well from surrounding objects:
House. . . . . Manure piles or other refuse. . . . .
Barns. . . . . Dumping grounds for kitchen slops. . . . . . .
Privy. . . . . Pig pens. . . . .
Slope of land relative to well. . . . .
Why do you think the water is polluted?. . . . .
. . . .
Has the well given any trouble before?. . . . .
What was nature of trouble?. . . . .
Has the water been tested before?. . . . . Report. . . . .
Remarks: . . . .
. . . .

BACTERIA ISOLATED FROM WATER SAMPLES

Designation of Organisms.	Morphology	Gram stain	Pigment	Nitrites	Indol	Liq. of gelatin	Neut. red agar	Neut. red broth	Lit. lact. agar	Lit. milk	Potato	Con.-Drig. agar.	Vosg.-Prosk. react.	Esculin bile	$H_2 : CO_2$	Fermentation in										Patho-genesis.		
																Dextrose	Lactose	Saccharose	Dulcit	Adonit	Inulin	Mannit	Raffinose	Liver broth	Lact. bile	Mice	Guinea pigs	Rabbits

SOIL MICROBIOLOGY

EXERCISE 1. A COMPARATIVE STUDY OF THE NUMBER AND TYPES OF MICROORGANISMS IN SOIL AND MANURE

Apparatus. Soil borer or auger; tamper; shield for soil borer; small covered clean enamelled steamed pails; * large spatula; large 3 or 4 tined fork; 1.5 liter flasks containing 1000 c.c. sterile water; 90 and 99 c.c. dilution flasks (sterile water); tubes of Conn's asparaginate agar or of Brown's albumen agar; sterile 1 c.c. and 10 c.c. pipettes; sterile Petri dishes; glass-stoppered weighing bottles; balances; drying oven, 100° C.

Culture. Soil (different types); manure (different types).

It is suggested that each student use one type of soil and one type of manure for this experiment, and make comparisons of the results obtained among the different members of the class.

Method. **1.** If the soil is dry or loose tamp it until it is quite firm, then place the shield (see Neller's article) upon the surface of the soil to be sampled and push it vertically into the soil until the flange rests upon the surface.

2. Insert the soil auger or borer into the opening of the shield and remove soil to the depth desired. The shield enables subsoil to be sampled without becoming contaminated with surface soil. Place the soil obtained in the steamed covered pail and remove immediately to the laboratory. The pail should be dry inside and kept covered as moisture determinations are to be made on each sample. If a plot is to be sampled, a sufficiently large number of samples should be taken to be representative. Borings may be made at

* Sterilized paper bags or similar containers may be used if moisture determinations are not to be made on the samples.

different depths as desired; all borings of one set should be of the same depth.

3. At the laboratory, thoroughly and quickly mix the sample in the pail, using the flamed fork and spatula; place a representative sample of the soil in a tared glass-stoppered weighing bottle and determine the moisture present on the oven-dry basis.

4. Weigh 100 grams of the well-mixed sample on a piece of sterile paper and add to the flask containing 1000 c.c. sterile water. This makes a 1–10 dilution. A large amount

Fig. 54.—Showing the Comparatively Enormous Value of Organic Microbial Food which Should Enter the Soil. (Carver, Yearbook of U. S. Dept. of Agr., 1914.)

of soil is used to obtain a more representative sample and to reduce the error as much as possible.

Moisture determinations and the 1–10 dilution should be made similarly with the manure.

5. Next, make a 1–100 dilution as follows: Shake the 1–10 dilution thoroughly for five minutes, allow the coarser particles to settle and transfer 10 c.c. of the supernatant liquid to 90 c.c. of sterile water. Save the 1–10 dilution for further study.

6. Make and plate the following dilutions: 1–1,000, 1–10,000, 1–100,000, 1–1,000,000, 1–10,000,000, using one of the above named media or any other medium which has

been found to give maximum counts. For a large class it is suggested that certain samples be plated on two or more kinds of media recommended by different investigators, plating on ordinary agar for comparison.

7. Incubate plates at room temperature for at least a week.

8. Count the colonies, computing and recording the results in tabular form as number of bacteria per gram of *oven-dry* soil or manure. Compare with counts per gram of the original moist sample. Which is the better method of determining counts? Why? Would the counts per gram of air-dry soil be as satisfactory?

What dilutions were best for the type of soil used? For the manure? Did all soils give the same results? All manures?

9. Examine the predominating colonies in hanging drop, or stain and determine the morphological types present. Are cocci present? Spiral forms? Spore-formers? What is Conn's theory concerning spore-forming bacteria in soil? Are actinomycetes present? Are chromogenic forms noted? If so, what morphological types are they? Are the molds present the common forms heretofore studied? Are molds of any particular importance in soil?

10. How does the microflora of the soil examined compare both as to number and types with that of the manure analyzed? After comparing your results with those obtained by other students, tell which soils are most alike in their flora. Suggest a reason why. Is there any resemblance in the microflora of the different manures? Would organisms of the colon group be expected in all manures? What is known about the function of bacteria of this type in the soil?

11. The same day that the plates are made, examine the 1–10 dilution of the soil and of the manure in a hanging drop and draw the forms seen. Are any of them motile? If so, what types? What types of microorganisms found microscopically will not develop on these plates? Explain why.

What is the procedure for obtaining these types? What soil microorganisms are classified under "edaphon"?

12. Are numbers of microorganisms as determined above, and biochemical efficiency in soils closely related? What relation have numbers of bacteria in soil to depth of soil? to season? What effect does cultivation of soil have upon bacterial numbers? Types?

13. What types of protozoa are found in the soil? What interrelationship exists between these microscopic animal forms and bacteria? What is meant by "partial sterilization" of soil? By what means may it be accomplished? Where used? What effect does it have on the above mentioned interrelationship? Protozoa from soils and manures may be observed and studied as noted on p. 138 in this manual. What type of nutrient solutions are suitable for their cultivation?

14. Are unusual precautions necessary in taking soil samples for ordinary bacteriological tests? Name three different ways in which either surface or subsurface soil samples may be taken without contamination from utensils or from other portions of the soil.

15. Give a brief résumé of a microscopic method for counting bacteria in soils; in manure. What are the limitations in each case?

REFERENCES

Breed, R. S., and Dotterer, W. D.: The Number of Colonies Allowable on Satisfactory Agar Plates. Tech. Bul. No. 53 (1916), N. Y. (Geneva) Agr. Expt. Sta.

Brown, P. E.: Methods for Bacteriological Examination of Soils, Res. Bul. No. 11 (1913), Iowa Agr. Expt. Sta.

Conn, H. J.: Culture Media for Use in the Plate Method of Counting Soil Bacteria. Tech. Bul. No. 38 (1914), N. Y. (Geneva) Agr. Expt. Sta.

—— Bacteria of Frozen Soil. Tech. Bul. No. 35 (1914), N. Y. (Geneva) Agr. Expt. Sta.

—— The Microscopic Study of Bacteria and Fungi in Soil. Tech. Bul. No. 64, (1918), N. Y. (Geneva) Agr. Expt. Sta.

Conn, H. J.: Soil Flora Studies, I, II, III, IV. Tech Bul. Nos. 57, 58, 59 (1917), N. Y. (Geneva) Agr. Expt. Sta.

Francé, R. H.: Das Edaphon (1913), 99 pages.

Fred, E. B.: Laboratory Manual of Soil Bacteriology (1916), p. 97.

Jensen, C. N.: Fungous Flora of the Soil. Bul. 315, Cornell, N. Y. (1912).

King, W. E. and Doryland, C. J. T.: The Influence of Depth of Cultivation upon Soil Bacteria and Their Activities. Bul. No. 161 (1909), Kansas Agr. Expt. Sta.

Löhnis, F.: Laboratory Methods in Agricultural Bacteriology (1913), pp. 89–90.

Marshall: Microbiology (1917), pp. 302–312.

Neller, J. R.: A Soil Sampler for Bacteriological and Chemical Purposes. Soil Sci. Vol. 4 (1917), pp. 109–112.

Russell, E. J.: Complexity of the Microorganic Population of the Soil. Science 37, pp. 519–522.

—— and Hutchinson, E. B.: The Effect of Partial Sterilization of Soil on the Production of Plant Foods. II. Jour. Agr. Sci. V (1912–13), pp. 152–221.

Waksman, S. A.: Effect of Protozoa on Bacterial Activities in the Soil. Soil Science, II, pp. 363–376 (1916).

—— The Importance of Mold Action in the Soil. Soil Sci., VI (1918), pp. 137–155.

—— Bacterial Numbers in Soils at Different Depths and in Different Seasons of the Year. Soil Sci., Vol. I, (1916), pp. 363–380.

Whiting, A. L.: Soil Biology Laboratory Manual (1917), pp. 121–122.

Wyant, Z. N.: A Comparison of the Technic Recommended by Various Authors for Quantitative Bacteriological Analysis of Soil. Soil Sci., Vol. XI, 1921, pp. 295–303.

EXERCISE 2. A STUDY OF SOIL ACTINOMYCETES

Apparatus. Four Petri dishes; one 1,000 c.c., several 99 c.c., and 90 c.c. sterile dilution flasks; sterile 1 c.c. and 10 c.c. pipettes; twelve tubes of Conn's agar for actinomycetes; several tubes each of Dunham's solution, nitrate solution, starch agar, cellulose agar and fermentation tubes of sugar broths containing indicator; ordinary stains.

Culture. Sod.

Method. 1. Make a series of 4 dilution plates in Conn's agar for actinomycetes, using dilutions from 1–10,000 to

1–10,000,000 and incubate at 25° C. Is a special medium necessary for growing this type of microorganism?

2. Examine plate cultures macroscopically after three to six days. Compare the appearance of the colonies developing on this special agar with colonies developing (from any source) on ordinary agar plates; also with colonies developing on the special agar used for obtaining maximum counts in soil (Ex. 1, Soil Microbiology). Record these observations. In general how do actinomycetes colonies differ in appearance from bacterial colonies?

3. Test the consistency of several colonies with a sterile platinum needle. How do colonies of actinomycetes differ in this respect from bacterial colonies?

4. Tilt the cover of the Petri dish slightly and determine the odor. What descriptive term is used by various authors for the typical odor of soil actinomycetes? To what is this odor attributed?

5. Examine microscopically the different types of actinomycetes colonies. Are any of these organisms motile? How do they compare as to shape, size, arrangement, etc., with bacteria heretofore studied? Are spores present? Do they stain similarly to bacteria? What special stain has been found to be valuable as a differentiating agent?

Make several permanent preparations showing different morphological types. With what class of microorganisms do different recent investigators place the actinomycetes?

6. Count the actinomycetic colonies and estimate the number per gram of oven-dry soil. How do your results compare with those of Waksman and Curtis and other investigators? What is known of the comparative numbers of actinomycetes and bacteria in soil? Are the actinomycetes species found in manures?

7. Isolate the different predominating types of actinomycetes on slanted Conn's agar, and determine whether each can (*a*) ammonify, (*b*) reduce nitrates (*c*) produce H_2S, (*d*) ferment ordinary sugar, (*e*) attack starch and (*f*) cellu-

lose, (*g*) grow anaerobically. From the results obtained state the possible importance of each type in the soil. What seems to be the function of certain actinomycetes in the soil? What physiological characteristic seems to be common to the greater number of soil actinomycetes? Is this of any importance?

8. How do the proteolytic enzymes of certain actinomycetes compare with those of other soil fungi? Which carbohydrates are attacked most readily by a large number of species of the actinomycetes? How are actinomycetes apparently influenced by the presence of acid in the soil? When is this knowledge of economic importance?

9. Name a species of actinomycetes pathogenic to plants; name four species pathogenic to animals or man. What pathogenic actinomycetes have been found in the soil? What is known of the geographical distribution of this species?

REFERENCES

Besson, A.: Practical Bacteriology, Microbiology and Serum Therapy. Transl. by H. J. Hutchins (1913), p. 655–669.

Buchanan, R. E.: Studies in the Classification and Nomenclature of the Bacteria, VIII. The Subgroups and Genera of the *Actinomycetales*. Jour. Bact., Vol. 3 (1918), p. 403–406.

—— Veterinary Bacteriology, Second Edition (1916), p. 421–431.

Conn, H. J.: Agricultural Bacteriology, pp. 34, 70, 120.

—— A Possible Function of Actinomycetes in Soil. Tech. Bul. 52, N. Y. (Geneva) Agr. Expt. Sta. (1919).

—— Soil Flora Studies. V. Actinomycetes in Soil, N. Y. (Geneva) Agr. Expt. Sta. Tech. Bul., 50 (1917).

Drechsler, C.: Morphology of the Genus *Actinomyces*, I, p. 65–83: II, p. 147–169. Bot. Gaz., Vol. 67 (1919).

Eyre: Bacteriological Technic, Second Edition, pp, 470–478.

Marshall: Microbiology, Second Edition, pp. 289–314.

Waksman, S. A.: Studies on the Proteolytic Enzymes of Soil Fungi and Actinomycetes. Jour. Bact., 3 (1918), p. 509–529.

—— Studies in the Metabolism of Actinomycetes, Part I, p. 189–216, Part II, p. 307–330. Jour. Bact., Vol. 4 (1919).

—— Cultural Studies of Species of *Actinamyces*. Soil Sci., VIII (1919), pp. 71–207, 4 pls.

Waksman, S. A., and Curtis, P. E.: The Actinomyces of the Soil. Soil Sci., I (1916), pp. 99–143.
—— The Occurrence of Actinomycetes in the Soil. Soil Sci., VI (1918), pp. 309–320.

EXERCISE 3. A DEMONSTRATION OF THE ACTIVITY OF AEROBIC CELLULOSE DECOMPOSING ORGANISMS FOUND IN SOIL AND MANURE

Apparatus. Twelve Petri dishes; twenty-four pieces of round filter paper to fit Petri dishes; sterile 0.05% K_2HPO_4; $MgNH_4PO_4$; sterile 0.05% NH_4NO_3; sterile water; sterile 1 c.c. and 10 c.c. pipettes; 90 c.c. and 99 c.c. dilution flasks; forceps.

Culture. Soil rich in humus, and well-rotted manure.

Method. **1.** Weigh the filter papers in pairs, to the tenth of a gram.

2. Place a thin layer of $MgNH_4PO_4$ between the pair of weighed filter papers in each Petri dish and sterilize in the hot air oven or autoclav.

3. Make dilutions of 1–1,000, 1–10,000 and 1–100,000 of the soil and manure, and inoculate the Petri dishes in duplicate, distributing well 1 c.c. of each dilution over the surface of the filter paper. Save all dilution flasks for use in Exercise 4.

4. Thoroughly moisten the paper in each dish of both sets of dilutions with sterile 0.05% K_2HPO_4 solution; moisten one set only of the soil and of the manure with 0.05% NH_4NO_3.

5. Keep plates at 25° to 30° C. in a moist chamber, or moisten every other day with sterile water to prevent drying out. The total incubation period should be two weeks at least.

6. In three to six days, yellow to brown spots should appear. In time, thin places can be detected by holding the plate up to the light, and later holes will be formed. As soon

as these characteristic spots appear, with a sterile platinum needle, test the consistency of the paper in the spots which appear to be attacked and compare with that of the undecomposed filter paper. Describe the results.

7. Determine microscopically by hanging drop or by staining, the morphological types of organisms present in each kind of spot on both sets of plates. What effect does the presence of NH_4NO_3 seem to have on the type of microflora developing?

Are cellulose-decomposing microorganisms found in the higher dilutions employed? What does this suggest?

8. At the end of two weeks, remove the filter papers from each set of plates with forceps, carefully wash, dry, weigh and record loss in weight with each dilution. Which inoculum contained the more active cellulose decomposers? What does this suggest? What products are formed in the aerobic decomposition of cellulose?

9. Name the aerobic cellulose-decomposing bacteria which have been described and identified by McBeth and Scales, and others. To which species do the bacteria on your plates correspond morphologically?

10. Examine microscopically the cellulose-decomposing molds present. Are you familiar with any of the species? What type predominates, if any?

11. What types of microorganisms only occasionally seen on cellulose plates are also very considerably concerned in the aerobic destruction of cellulose?

12. What steps would you take to isolate and determine the cellulose-decomposing power of the organisms growing on your plates?

Are the chemical elements used above present in the soil or manure in this or some other form? May other chemicals be used in this experiment? For what important processes in soil, etc., does cellulose serve as a source of energy?

13. Which will be more easily attacked in the soil, the

cellulose of a green crop or of a dry crop? Are cellulose-destroying organisms more numerous in fresh or in well-rotted manure? From what you know of the feeding habits of various farm animals, what would you predict as to the difference in the content of cellulose-decomposing organisms in their respective fresh manures? Would you expect to find cellulose decomposers in human feces?

From the manner in which different farm manures are handled for fertilizer purposes, which types would probably have the largest content of cellulose-decomposing organisms when ready for application to the land?

Are manures from the sea subject to cellulose decomposition when utilized on the farm lands?

14. Where besides in soil or manures are aerobic cellulose-decomposing organisms found? Are any of these of economic importance?

REFERENCES

Conn, H. J.: A Possible Function of *Actinomycetes* in Soil. Tech. Bul., 52 (1916), N. Y. (Geneva) Agr. Expt. Sta.

Fippin, E. O.: Farm Manure. Lesson 127, The Soil Series, The Cornell Reading Course for the Farm. (1917), N. Y. State College of Agr., Ithaca, N. Y.

Jenkins, E. H. and Street, J. P.: Manure from the Sea. Conn. (New Haven) Agr. Expt. Sta. Bul. 194 (1917),

Kellerman, K. F., McBeth, I. G., Scales, F. M., and Smith, N. R.: Identification and Classification of Cellulose-Dissolving Bacteria, Cent. f. Bakt. 2 Abt., Vol. 39 (1913–14), p. 502.

Lipman, J. G. and Brown, P. E.: Laboratory Guide in Soil Bacteriology (1911), pp. 62, 65.

Löhnis, F.: Laboratory Methods in Agricultural Bacteriology (1913), pp. 104–105.

Marshall: Microbiology, Second Edition, pp. 315–317.

McBeth, I. G. and Scales, F. M.: The Destruction of Cellulose by Bacteria and Filamentous Fungi. Bul. 266, B. P. I., U. S. Dept. Agr. (1913).

Scales, F. M.: Some Filamentous Fungi Tested for Cellulose-Destroying Power. Bot. Gaz., Vol. 60. (1915), pp. 149–155.

THOMAS, W.: Analyses of Domestic Manures and Related Substances. Ann. Rept. of Penn. State College Agr. Expt. Sta. for 1914–15, pp. 413–425.

WAKSMAN, S. A.: Soil Fungi and Their Activities. Soil Sci., Vol. 2 (1916), pp. 146–147.

EXERCISE 4. TO ILLUSTRATE THE ANAEROBIC DECOMPOSITION OF CELLULOSE BY SOIL AND FECAL ORGANISMS

Apparatus. Large tubes of Omelianski's synthetic medium for anaerobic cellulose fermentation containing respectively weighed pieces of filter paper, and wheat or oat straw; tubes of sterile white paraffin oil; dilution flasks containing the dilutions used in Exercise 3; lead acetate paper.

Culture. Dilutions of rich soil; sand or air-dried soil; fresh and decayed manure.

Method. **1.** Inoculate a tube of Omelianski's medium containing each kind of cellulose with each dilution used in Exercise 3.

2. Inoculate one tube each with a small portion of fresh manure, and another with poor soil such as sand, or with air-dried soil.

3. Cover the medium in each case with about 1 cm. of sterile paraffin oil, insert a strip of moistened lead acetate paper beside the cotton plug and incubate at 30°–35° C. for four to six weeks.

4. Examine every week or so and record any changes occurring in the filter paper. In what dilutions of soil is the cellulose decomposition most active? How do these results compare with those of the decayed manure? Is gas formed in any case? If so, what gas or gases may these be?

5. When the filter paper or straw in any tube shows marked signs of disintegration make transfers to new tubes of the synthetic medium, cover with oil, incubate as before, and note increased rapidity of fermentation. Which shows

the more rapid decomposition, straw or pure cellulose? What are the chief products formed?

6. Examine the organisms causing the disintegration of the filter paper and straw in hanging drop and make permanent stains. Are as many morphological types present as in aerobic cellulose decomposition?

7. When marked decomposition has taken place in the original tube, filter the contents of the tubes upon weighed filter paper, wash with water, dry, and weigh. Record the loss of weight in each case. From the data thus obtained calculate how many pounds of straw per acre would be decomposed under anaerobic conditions in five months at the rate found in this experiment.

8. Starch, cotton, peat, etc., may be substituted for filter paper or straw in this medium and its digestion studied in the same way.

Not taking soil into consideration, where do anaerobic cellulose-decomposing organisms probably play a most important part in nature? In what industries are they of economic importance?

9. The microorganisms favored by this synthetic medium have only in exceptional cases been grown on solid media. How can these types be separated?

10. Is hydrogen sulfid formed? What effect do certain species of these microbes have on nitrates?

11. What beneficial purposes may cellulose serve under anaerobic conditions?

Why may an excess of straw or coarse manure be very often injurious to soil? Which appears to be the more valuable process to foster in the manure or compost heap, anaerobic or aerobic cellulose decomposition? Why? For what three processes does cellulose serve as a source of energy under anaerobic conditions?

12. Data and observations should be given in full. Draw any conclusions warranted and indicate any practical applications that may be made.

REFERENCES

Kellerman, K. F., McBeth, I. G., Scales, F. M., and Smith, N. R., *l. c.* Exercise 3.

Löhnis, F.: Laboratory Methods in Agricultural Bacteriology (1913), p. 93.

Löhnis, F.: Vorlesungen über landwirtschaftliche Bakteriologie (1913), pp. 171–176.

Marshall: Microbiology (1917), pp. 167, 277–278, 315–317, 555, 558.

McBeth, I. G. and Scales, F. M., *l. c.* Exercise 3.

Russell, E. T., and Richards, E. H.: The Changes Taking Place During the Storage of Farmyard Manure. Jour. Agr. Sci., Vol. 8 (1917), Part IV, pp. 495–563.

Whiting, A. L.: Soil Biology Laboratory Manual (1917), pp. 40–41.

EXERCISE 5. A STUDY OF NITRIFICATION IN SOIL AND MANURE

Apparatus. Eight sterile deep Petri dishes; spatula; at least 500 c.c. of sterile water; sterile pipettes; $(NH_4)_2SO_4$; dried blood; eight clean 500 c.c. flasks.

Culture. Fertile soil, and manure from surface of heap, both air-dry.

Method. 1. Weigh into deep Petri dishes six 100 gram portions of air-dry soil, and two similar portions of air-dry manure.

2. Add to the soil, ammonium sulfate and dried blood as follows:

Nos. 1 and 2—soil alone,
Nos. 3 and 4—soil + 0.1 gm. $(NH_4)_2SO_4$,
Nos. 5 and 6—soil + 0.2 gm. dried blood,
Nos. 7 and 8—manure,

stirring in the additions thoroughly with a sterile spatula.

3. Add to each dish sufficient sterile water to bring the moisture conditions to the optimum, placing the same amount in each dish.

4. Record total weight of each dish with cover.

5. Prepare a third set of samples like Nos. 1, 3, 5, and 7 and test immediately as follows: add 250 c.c. of distilled water to each and stir thoroughly over a period of an hour, allow to settle and filter each into a clean flask. Wash the soil on the filter with distilled water and make up to 400 c.c.

6. Test the filtrate in each case for nitrites, nitrates, and ammonia (for methods see Exercise 45, Part I), always testing the distilled water at the same time for a control.

7. Tabulate results obtained colorimetrically by use of the following symbols: absence of color (as compared with control), $-$; small amount, $\pm$; decided, $+$; more decided, $++$; excessive, $+++$. The duplicate samples may then be discarded.

8. Weigh each dish once a week, restoring the loss of moisture with sterile water and stirring the soil thoroughly with a sterile spatula to maintain aerobiosis.

9. Incubate four weeks at 25° to 28° C.

10. At the end of this period, test each sample for nitrites, nitrates, and ammonia and record results as directed under paragraphs 5 to 7 of this Exercise. Compare results with those obtained from the original samples. Which of these nitrogenous decomposition products is assimilable by plants?

11. Explain the decomposition taking place in each case giving the successive steps in the disintegration of the nitrogenous matter present and writing the theoretical chemical reactions where possible.

Indicate how many distinct steps occur in this decomposition.

By what organism is each initiated? What interrelationships exist between these organisms?

12. What different methods may be employed for isolating these nitrifying bacteria? What is the principle of each method? Nitrifying bacteria do not grow on the ordinary solid media. What peculiarity characterizes the composition of the various media used for their cultivation and isolation?

13. Do nitrites exist in normal soils? What influence does the amount and distribution of moisture have on nitrification? What substances retard and check nitrification and in what concentrations? What substances accelerate this process? Of what importance is the nitrification process from the standpoint of the liberation of insoluble minerals?

What methods does the agriculturist use which aid in conserving these organisms? At what depths does nitrification take place?

Where are nitrifying organisms found besides in soil? What is their significance in each case?

Give one or more instances in history in which the microbial nitrification process gained great importance.

REFERENCES

Fred, E. B.: Laboratory Manual of Soil Bacteriology (1916), pp. 47–54, 100–106.

King, F. H.: Farmers of Forty Centuries (1911), pp. 251–254.

Lyon, T. L., Fippin, E. O., and Buckman, H. O.: Soils. Their Properties and Management (1915), pp. 447–454.

Marshall: Microbiology (1917), pp. 146, 174, 275–277, 281, 285, 327–333.

Voorhees, E. B., and Lipman, J. G.: A Review of Investigations in Soil Bacteriology. Bul. 194, U. S. D. A. (1907), pp. 57–67.

EXERCISE 6. ILLUSTRATION OF THE ACTIVITIES OF DENITRIFYING BACTERIA IN SOIL AND IN THE MANURE HEAP.

Apparatus. Twelve sterile deep Petri dishes; sterile water; KNO_3 crystals and 1% sterile solution; sterile spatula; tubes of sterile Giltay's agar and nitrate agar; fermentation tubes of nitrate broth alone and containing 1% dextrose, and of Giltay's solution; tubes of Dunham's peptone solution.

Culture. Cultivated soil; manure from anaerobic portion of heap; *B. coli*; *B. fluorescens liquefaciens*.

Method. **1.** Weigh out eight 100 gram samples of soil and four of manure into the sterile deep Petri dishes.

2. Prepare soil and manure as follows, using sterile water in every case:

Nos. 1 and 2 soil, half saturated,
Nos. 3 and 4 soil, saturated,
Nos. 5 and 6 soil, +0.06 gm. KNO_3, half saturated,
Nos. 7 and 8 soil, +0.06 gm. KNO_3, saturated,
Nos. 9 and 10 Manure, half saturated,
Nos. 11 and 12 Manure, saturated.

3. In dishes Nos. 1 and 2, and 5 to 10 thoroughly incorporate the water and potassium nitrate where indicated.

4. Prepare a third set of samples like Nos. 3, 7, and 11 using 250 c.c. distilled water for each, thus supersaturating the sample. Stir thoroughly and proceed with determinations of nitrate, nitrite and ammonia on the fresh samples according to paragraphs 5 to 7 of Exercise 5, Soil Microbiology.

5. Incubate samples of soil and manure at 28°–30° C. for two weeks.

6. At the end of this time make anaerobic loop dilution plates in Giltay's agar and also make similar dilutions in nitrate broth fermentation tubes (cover with sterile paraffin oil) from one dish of each pair; incubate at 30° C.

7. Record gas-formation in either medium in forty-eight hours or as soon as formed, in the dilutions in which it occurs. What is this gas?

8. From the tube showing the most vigorous gas-formation or from the plate showing isolated colonies of gas-formers isolate the predominant gas-producers in pure culture using nitrate or Giltay's agar for plating, then test a portion of the liquid culture medium for nitrates and record results.

9. Compare the action in fermentation tubes of Giltay's solution and in nitrate broth, both with and without sugar (1% dextrose), of pure cultures of the denitrifying organisms isolated, with *B. coli* and *B. fluorescens liquefaciens*. Deter-

mine the amount and nature of the gas formed in each tube and compare results. Determine the presence of N_2 by elimination, i. e. by testing for CO_2 and H_2 (See p. 116). From the results obtained, is it suggested that organisms of the colon and green liquefying type may be of importance in soils or in compost heaps? What influence does dextrose have upon the amount and rate of gas-formation?

10. Test one sample of each pair at this time for nitrates, nitrites, and ammonia as before and record results.

11. If it is desired to calculate the actual loss of nitrogen in the samples, the total nitrogen must be determined (by the Kjeldahl method) on the duplicate samples before and after the two weeks' incubation period.

12. Inoculate in duplicate, tubes of peptone solution with the most active denitrifier isolated.

13. Examine every day or so for growth and gas-formation if any. Record results. At the end of a week, test cultures for nitrates, nitrites, and NH_3. Is this organism a strict denitrifier or does it reduce organic nitrogen as well?

14. What is the physiological significance of nitrate reduction? Name several important denitrifiers. Are denitrifying organisms numerous in soil? in manure? Under what conditions are they most numerous? What type of manure contains the greater number of denitrifiers? Why is this so? Do any methods of storing manure encourage denitrification? If so, what are they? What effect respectively have fresh and partly decomposed organic matter upon denitrification in soil? What types of soil are most liable to foster denitrifying organisms? Why? In what way does this knowledge of the physiological characteristics and requirements of the denitrifying organisms determine certain agricultural practices?

REFERENCES

Conn, H. W.: Agricultural Bacteriology, Third Edition (1918), pp. 73–75, 79.

Ferguson, M. and Fred, E. B.: Denitrification. The Effect of Fresh and Well Rotted Manure on Plant Growth. Ann. Rept. of Va. Agr. Expt. Sta. 1908. pp. 134–149.
Russell, E. J.: Soil Conditions and Plant Growth (1912), pp. 98, 99.
Voorhees, E. B. and Lipman, J. G.: A Review of Investigations in Soil Bacteriology. Bul. 194, U. S. D. A. (1907), pp. 68–75.

EXERCISE 7. DEMONSTRATION OF SULFOFICATION IN SOIL

Apparatus. Two deep Petri dishes; quartz sand of medium fineness; about 50 c.c. sterile solution composed of 0.1% $NaHCO_3$, 0.01% NH_4Cl and 0.01% $MgCl_2$ in tap water; sterile 5 c.c. pipette with large delivery opening; sterile 90 c.c. dilution flask; N/10 barium chloride solution; sulfur; tri-basic calcium or rock phosphate; strips of lead acetate paper; distilled water; round filter paper 5 to 6 mm. diameter; small funnel 3 to 4 mm. diameter.

Culture. Fertile soil.

Method. 1. Place 100 gms. of clean, well washed dry quartz sand in each Petri dish and add to each dish 0.1 gm. each of sulfur and of $Ca_3 (PO_4)_2$ or rock phosphate.

2. Mix well while dry, then add 20 c.c. of the above nutrient solution, distributing it well over the surface of the sand and sterilize in the autoclav.

3. Add 5 c.c. of a 1–10 dilution of the fertile soil to one dish when cool, distributing the inoculum over the entire surface of the sand. Leave the other dish uninoculated for control. Place a moistened strip of lead acetate paper underneath the cover of each to detect the formation of H_2S.

4. Incubate both dishes at 25°–30° C. for four weeks at least.

5. At the end of the incubation period add 50 c.c. of distilled water to each dish, stir thoroughly and allow to stand for a few minutes.

6. Filter some of this supernatant liquid and to 5 c.c. of

the filtrate from the inoculated dish add with a pipette tenth normal barium chloride solution till no more precipitate is formed. What is this precipitate? From what is it formed? Repeat this procedure with the filtrate from the uninoculated dish. Is the same phenomenon noted in this case?

7. Carefully weigh a small round filter paper, shake the tube containing the precipitate to get it all into suspension, filter upon the weighed filter paper and weigh again when dry.

Approximately what proportion of the sulfur has been attacked by the soil microbes? From the data obtained calculate how much sulfur applied at the rate of 100 pounds per acre would be oxidized in three months. What is known of the activity of sulfofiers in the compost heap?

8. What effect has the sulfofication process been demonstrated to have upon insoluble mineral phosphates? Does the insoluble phosphate affect sulfofication? If so, how? What is the economic importance in either case? What chemicals containing no phosphorus could be used to supplant insoluble phosphates? What would be their purpose in this case?

What is the optimum moisture content for sulfofication? What effects does an excessive water content of soil have upon sulfofication? Too little water?

In what agricultural practices can the knowledge gained in this exercise be applied?

9. Give results and any conclusions in detail. Point out any possible practical applications.

REFERENCES

Brown, P. E. and Gwinn, A. R.: Effect of Sulfur and Manure on Availability of Rock Phosphate on Soil. Iowa Agr. Expt. Sta. Res. Bul. 43 (1917).

Lipman, J. G. and McLean, H. C.: Experiments with Sulfur-Phosphate Composts Conducted under Field Conditions. Soil Sci. V (1918), 243–250.

Lipman, J. G., McLean, H. C. and Lint, H. C.: The Oxidation of Sulfur in Soils as a Means of Increasing the Availability of Mineral Phosphates. Soil Sci. I (1916), pp. 533–540.

—— Sulfur Oxidation in Soils and Its Effect on the Availability of Mineral Phosphates. Soil Sci. II (1916), pp. 499–538.

Shedd, O. M.: The Relation of Sulfur to Soil Fertility. Kentucky Agr. Expt. Sta. Bul. 188 (1914).

EXERCISE 8. ISOLATION AND STUDY OF *AZOTOBACTER*, THE NON-SYMBIOTIC AEROBIC NITROGEN-FIXING ORGANISM OF SOIL

Apparatus. Tubes of mannit agar; 4 stérile 100 c.c. Erlenmeyer flasks containing 50 c.c. sterile mannit solution; concave slides; cover-glasses; filter paper; saturated alcoholic gentian violet; ordinary aqueous-alcoholic stains; Lugol's iodin solution.

Culture. Clay loam; sandy loam; manure.

Method. **1.** Inoculate flasks of mannit solution as follows:

Flask 1—nothing, control.
Flask 2—0.1 gm. clay loam.
Flask 3—0.1 gm. sandy loam.
Flask 4—0.1 gm. manure.

2. Incubate at room temperature and note the changes taking place. A wrinkled skin, white at first, becoming brownish later, especially around the edge, is gradually formed. This is composed of the cells of *Azotobacter* sp. What species produce characteristic brown pigment? What conditions favor pigment production? Is it intra- or extracellular? Do all species produce brown pigment? Are pigments of other colors formed by any species of *Azotobacter*?

3. From time to time examine the cultures in the hanging drop and note the type of organisms predominating. Frequently single, small, thin bacilli are visible between the

large cells of *Azotobacter.* These are almost always *B. radiobacter,* a type resembling the nodule bacteria *B. radicicola,* and can also fix nitrogen to a slight extent. The *Azotobacter* species are the most vigorous aerobic free-nitrogen-fixing organisms yet discovered. (See reference, E. B. Fred, Exercise 8, Soil Microbiology.)

Fig. 55.—*Azotobacter.* x1000; Smear from Six-day Old Culture on Ashby's Agar at 25° C. Showing organisms and capsules in various stages of development. (Dan H. Jones.)

What is the name of the best known anaerobic nitrogen-fixing organism? What action in soil may be counter-balanced, in part at least by the activities of this latter type of nitrogen-fixing organism? How important is this fixation under normal conditions? Does this type form endospores? What statement is made by Winogradski regarding the growth of the anaerobic nitrogen-fixing bacillus under aerobic conditions? State other cases which suggest that this principle is generally applicable. What is known of the distribution of this anaerobic type? Of the *Azotobacter* species?

4. When the surface film or ring becomes brownish make plates from this portion of the culture in mannit agar using relatively high dilutions, and incubate at room temperature. If it is desired to test nitrogen-fixation, total nitrogen must be determined on the original solution and on the old culture by the Kjeldahl method.

5. After about a week, examine the organisms in the various colonies and isolate *Azotobacter chroococcum* if possible, placing it on a mannit agar slant. A sand slope may also be used. How is it prepared? *Azotobacter* species are

very frequently difficult to separate from *B. radiobacter* on account of the slimy cell walls of the former bacteria. The quickest method of separation is to reinoculate first into the mannit solution, allow to grow for several days, and then reisolate.

6. Is *B. radiobacter* present on the plates along with *Azotobacter* or in isolated colonies? Isolate and study pure cultures of this organism, comparing it morphologically and culturally with *Azotobacter*. How does *B. radiobacter* compare with *B. radicicola* culturally?

7. Save several of the plates having well-isolated *Azotobacter* colonies and note any changes which may occur. To what are these due?

8. If any brown colonies develop make smears directly from the colony without the use of water, stain with saturated alcoholic gentian violet and examine under the oil immersion lens. Measure the stained bacteria. How large are the capsules? Stain and examine young colonies with this same stain. How does the age of the colony affect the staining properties of the organisms of which it is composed? What stains are most satisfactory for this organism? Flood smears of both old and young cultures with Lugol's iodin solution and examine under oil. What is shown in each case?

9. Does *Azotobacter* form spores? involution forms? gonidia? Is it influenced by desiccation? By any other physical influences?

10. What farm practices favor the development of the *Azotobacter*? In what way? What soil conditions are favorable to this species? These conditions are favorable to what other bacteria of economic importance? Are these same soil conditions favorable to plants? To what plants may they be unfavorable?

Would it pay in any case to inoculate soil with *Azotobacter*?

11. How does the pH value of the mannit solution used compare with that of soils in which *Azotobacter* will grow?

What pH limit in the acid range has been found for *Azotobacter* by one or more investigators? Of what importance is this? How much nitrogen will *Azotobacter* species fix per gram of carbohydrate? How does this amount compare with that fixed by the anaerobic species?

12. State your results in full and draw conclusions. Point out the practical applications of the above.

REFERENCES

Ashby, S. F.: Some Observations on the Assimilation of Atmospheric Nitrogen by a Free Living Soil Organism, *Azotobacter Chroococcum* of Beijerinck. Jour. Agr. Sci. (1907), Vol. 2., pp. 35–51.

Bonazzi, A.: Cytological Studies of *Azotobacter Chroococcum.* Jour. Agr. Res. (1915), Vol. 4, pp. 225–239, 3 plates.

Emerson, P.: Soil Inoculation With *Azotobacter.* Res. Bul. 45 (1918), Iowa Agr. Expt. Sta.

Gainey, P. L.: Soil Reaction and the Growth of *Azotobacter.* Jour. Agr. Res., Vol. 14 (1918), pp. 265–272.

Hazelhoff, E. and Bredmann, G.: Investigations on Anaerobic Nitrogen-Collecting Bacteria. Landw. Jahrb. 35 (1906), No. 3, pp. 381–414. Abstract in Ex. Sta. Rec., Vol. 18 (1906–7), p. 429.

Hoffman, C. and Hammer, B. W.: Some Factors Concerned in the Fixation of Nitrogen by *Azotobacter.* Res. Bul. 12, Univ. of Wisconsin (1910).

Jones, Dan H.: A Morphological and Cultural Study of Some *Azotobacter.* Cent. f. Bakt., II Abt., Bd. 38 (1913), pp. 12–25, 5 plates.

—— Further Studies with Some *Azotobacter.* Cent. f. Bakt., II Abt., Bd. 42, pp. 68–69.

Lipman, J. G.: *Azotobacter* Studies. N. J. Agr. Expt. Sta. Rept. (1908), pp. 137–143.

Löhnis, F. and Smith, N. R.: Life Cycles of the Bacteria. Jour. Agr. Res., Vol. 6 (1916), pp. 675–702, 7 plates.

Marshall: Microbiology (1917), pp. 151, 293–294, 317, 319, 336, 339–343, 353, 357, 358, and 362.

Waksman, S. A.: The Occurrence of *Azotobacter* in Cranberry Soils. Sci. N. S., Vol. 48, (1918), pp. 653, 654.

Winogradski, S.: Assimilation of the Gaseous Nitrogen of the Air by Microbes. Compt. Rend. 118 (1894), No. 7, pp. 353–355. Abst. in Ex. Sta. Rec., Vol. 5 (1893–4), pp. 1010–11.

EXERCISE 9. A STUDY OF B. RADICICOLA, THE SYMBIOTIC NITROGEN-FIXER

B. radicicola lives in symbiosis with certain members of the legume family, but strains of this organism may be able to form nodules and live symbiotically on the roots of plants not belonging to this family. For this exercise, nodules from red clover, alfalfa or sweet clover are best adapted.

A. Isolation of *B. radicicola* from nodules.

Apparatus. Spade or trowel; sterile Petri dishes; tubes of nitrogen-poor ash agar; Congo red agar; tumbler; sterile filter paper; small forceps; scalpel or chisel-edged inoculating needle; platinum loop; clean slides; 1–500 mercuric chloride; 95% alcohol; tubes of sterile water; aqueous-alcoholic stains; eosin; Lugol's iodin solution.

Culture. From nodules of red clover, alfalfa or sweet clover.

Method. **1.** Using the spade or trowel, obtain the roots of one of the legumes mentioned which show nodule formation. Do not forcibly pull up the legume, as this procedure strips off most of the nodules, which develop almost without exception on the young rootlets.

Note.—Legumes having nodules may be stored for winter use in cool, moist earth.

2. Thoroughly wash the roots and place in clean, cool water.

3. Compare the size, numbers and location of the nodules on the roots of the different legumes.

4. Remove a good sized nodule from the roots, wash in clean water and immerse for three minutes in 1–500 mercuric chloride.

5. Place a drop of sterile water in each of three sterile Petri dishes.

6. Remove the nodule from the mercuric chloride solution with sterile forceps, take up the excess of the solution

Fig. 56.—Alfalfa Plants from Inoculated and Uninoculated Seed. (Orig. Northrup.)

between folds of sterile filter paper, then dip into alcohol the last traces of which should be removed by passing the nodule quickly through the flame.

7. Place the nodule on a flamed and cooled slide, and holding in sterile forceps cut it open by means of the sterile scalpel or chisel-edged needle.

8. Thrust a sterile platinum needle into the nodule in the middle of the newly-exposed surface and gently rotate the needle so that some of the contents or crushed tissue adheres to it.

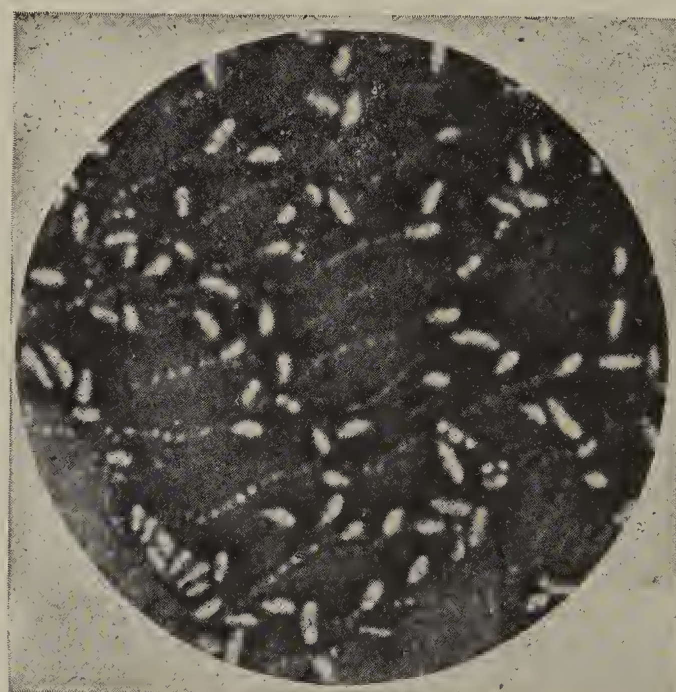


Fig. 57.—*Ps. radicicola,* Polar flagella, x1500. Twenty-five day old culture from sweet pea. (B. Barlow).

9. Transfer this material to the drop of sterile water in one of the sterile Petri dishes and make loop dilutions from this drop into the second and third Petri dishes.

10. Pour the plates, using tubes of nitrogen-poor ash agar and incubate at room temperature for three or four days.

11. Make a smear on a clean slide from the freshly cut surface of the nodule, stain and examine microscopically. What is the morphology of the organisms found in the nodules? What are "bacteroids"?

12. Make another smear directly from this same nodule, fix and stain with eosin followed by Lugol's iodin solution. What is demonstrated respectively with eosin and the iodin solution?

13. After a few days of incubation the colonies of *B. radicicola* will be noted on the plates as round, grayish-white-translucent, viscid drops, finely granular.

Various strains of *B. radicicola* vary markedly in their rate of growth, sliminess, and other physiological characteristics.

14. Examine these colonies in the hanging drop. The normal, short, rod forms should be present. These are actively motile during the first few days of growth.

15. Isolate several pure cultures of *B. radicicola* on slants of nitrogen-poor agar. Why is nitrogen-poor agar used for the cultivation of *B. radicicola?* Will this organism grow on mannit agar?

16. Make permanent stains of pure culture and compare with organisms on stained smear from nodule as to size, shape, etc. Are forms other than short rods present in either preparation?

Do all species of legumes or other plants show organisms of the same general morphology in the respective nodules? In pure culture?

17. Sometimes the roots of leguminous plants show, instead of the normal nodules, lesions of crown gall caused by *Bact. tumefaciens* which somewhat resemble *B. radicicola.*

For a rapid diagnosis, plate in Congo red agar. In this medium *B. radicicola* forms white colonies while *Bact. tumefaciens* absorbs the dye producing red or reddish colonies.

18. What common members of the legume family, under certain conditions, have their economic value raised by their symbiotic relations with *B. radicicola*? Name three plants not belonging to the *Leguminosæ* whose roots may bear nodules possibly caused by this organism.

B. Test of the Nodule-Forming Ability of *B. radicicola.*

There are several distinct strains of *B. radicicola* each of which is distinguished by its ability to invade and produce nodules on the roots of a certain fairly-well defined group of

genera in the *Leguminosæ*. There are at least ten of these groups, among the members of one group of which cross-inoculation readily takes place.

For instance, *B. radicicola* obtained from alfalfa nodules will infect sweet clover and vice versa, but this particular strain will not cause nodule formation on soy beans, on peas, etc.

The nodule-forming ability of the pure culture just isolated is to be compared with that of a stock culture of the same strain.

Apparatus. Ten large test tubes on foot, or liter Erlenmeyer flasks containing sterile nitrogen-poor agar (0.65% agar) to the depth of about 5 cm.; sterile tubes of nitrogen-poor agar; sterile Petri dishes; 1–500 mercuric chloride; sterile pipette; forceps; wash bottle containing sterile distilled water; seeds of the same legume as used in Part **A** of this exercise.

Culture. Two cultures of *B. radicicola*, the strain just isolated and the stock laboratory strain, both specific for the legume used in **A**.

Method. **1.** Soak the seeds of the legume in 1–500 mercuric chloride for five minutes.

2. Wash off the disinfectant with sterile distilled water, using sterile forceps, then follow Procedure I or II.

Procedure I

3. Place the seeds between layers of moist sterile filter paper in a Petri dish to germinate.

4. Transfer six to ten germinated seeds to the culture tubes. If larger seeds are employed use three to six only.

Keep in a well-lighted room for a few days. Examine the tubes and reject all that contain molds or other organisms.

5. Using a sterile pipette, inoculate four of these tubes with (*a*) a specific pure culture isolated in Part **A** of this exercise, and (*b*) a second set of four with the laboratory stock culture of this specific strain, by dropping upon the

seeds and surface of the agar a heavy suspension of the culture in sterile water.

Procedure II

3a. Inoculate the melted agar at 40° to 45° C. with the culture.

4a. Transfer several of the sterilized seeds to each of the culture tubes, using the numbers suggested in Procedure I according to the size of the seeds.

5a. Put the tubes containing the ungerminated seed in a warm place (30° to 35° C.) until the seeds germinate. Then follow as in Procedure I.

6. Keep two uninoculated tubes as controls.

7. Label the culture tubes and place them where they will be sufficiently protected from the sun, heat, cold, etc. *This is very important.* A piece of cheese cloth thrown over the tubes will protect them from the sun.

8. In two weeks to a month examine all culture tubes for nodules.

9. Record the presence, number, size, and shape of nodules, place of formation, etc., and show culture tubes containing nodule-bearing seedlings to instructor. Which culture was the more active in causing nodule formation? Why?

10. Isolate *B. radicicola* from one of these nodules and thus complete the cycle. If all of these operations are successful Koch's postulates have been fulfilled. What are Koch's postulates? With what organism were they first worked out?

11. What different methods have been employed commercially with a large degree of success for inoculating legumes on a practical scale? What are the advantages of each method? What methods failed and why? To what may poor results from artificial inoculation be attributed?

12. Does *B. radicicola* fix nitrogen in the absence of the host plant? If so, how much? Does the host plant ever resist the invasion of this organism?

In what form is the nitrogen fixed by *B. radicicola* supposed to be supplied to the plant?

13. What other methods may be employed in the laboratory for obtaining nodules experimentally?

REFERENCES

Burrill, T. J. and Hansen, R.: Is Symbiosis Possible Legume Bacteria and Non-legume Plants? Ill. Agr. Expt. Sta. Bul. 202, 1917.

Edwards, S. F. and Barlow, B.: Legume Bacteria, Bul. 169, Ontario Dept. of Agriculture (1909).

Fred, E. B.: A Physiological Study of the Legume Bacteria. Ann. Rept. Va. Polytechnic Institute (1911–12), pp. 145–173.

Kellerman, K. F.: Nitrogen-Gathering Plants, U. S. Dept. of Agr. Yearbook for 1910, pp. 213–218.

—— The use of Congo red in culture media. Cir. 130, Bur. Plant Ind., U. S. D. A., 1913.

Marshall: Microbiology (1917), pp. 151, 212, 242, 298, 341, 343–353.

Russell, E. J.: Soil Conditions and Plant Growth (1912), pp. 95–98.

Smith, E. F.: Bacteria in Relation to Plant Diseases, Vol. II, pp. 96–138.

EXERCISE 10. TO DEMONSTRATE THE CHANGE OF INSOLUBLE PHOSPHATES TO A SOLUBLE FORM THROUGH THE AGENCY OF MICROORGANISMS

Apparatus. Dextrose; di- or tri-calcium phosphate; tubes of soil-extract agar containing 2% dextrose; four 100 c.c. Erlenmeyer flasks.

Culture. Soil.

Method. 1. Place 0.1 gm. of di- or tri-calcium phosphate, and 60 c.c. of a 2% solution of dextrose in tap water in each flask. Sterilize.

2. To two flasks add 0.1 gm. soil each, leaving two for controls.

3. Incubate at 37° C., and after the fermentation has continued for some days, make plates from the inoculated flasks as follows:

4. Sterilize about 0.1 gram of di- or tri-calcium phosphate in each of three test tubes.

5. Place the contents of each tube in a sterile Petri dish; make loop-dilution plates from flasks in soil extract agar containing 2% dextrose, being careful to mix the phosphate well with the agar in the dish by carefully tilting.

6. Incubate at 37° C.

7. Note frequently the appearance of the plates. The colonies of acid-producing bacteria developing at this temperature dissolve the phosphate and thus become surrounded by a clear area similar to that produced by lactic acid-producing bacteria on dextrose calcium carbonate agar.

8. Examine the colonies in a hanging drop for morphology, motility, etc.

9. How is the action noted in **7** made use of practically?

In what compounds is phosphorus found in soil? Are these available as plant food? What are the functions of bacteria in this connection?

What relation has phosphorus to decay and nitrogen fixation?

10. Give results and any conclusions in detail. Point out any possible practical applications.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 257, 258.

Löhnis: Laboratory Methods in Agricultural Bacteriology, pp. 115–116.

Sackett, Patten and Brown: The Solvent Action of Soil Bacteria upon Insoluble Phosphates, etc., Spec. Bul. 43, Mich. Expt. Sta., 1908.

DAIRY MICROBIOLOGY

EXERCISE 1. A COMPARATIVE STUDY OF THE NUMBER AND TYPES OF MICROORGANISMS AND OTHER CELLS IN MILK

A. PLATING METHOD

Apparatus. Sterile Petri dishes; sterile 1 c.c. and 10 c.c. pipettes; tubes of sterile litmus lactose agar; 90 c.c. and 99 c.c. dilution flasks; tubes of sterile litmus milk.

Culture. Fresh milk from any source desired.

Method. **1.** Shake the milk vigorously one hundred times to obtain a homogeneous sample, and plate in dilutions, 1–100, 1–10,000 and 1–1,000,000 in litmus lactose agar.

2. Incubate the plates inverted at room temperature for five days.

3. Count at the end of this time, estimate the average number of bacteria per cubic centimeter and approximate the numbers of the different types of colonies.

4. Are acid colonies present? Chromogenic colonies? *B. subtilis* or *B. mycoides?*

What do the types signify?

5. Isolate the different types in litmus milk and note their action. To which group of microorganisms does each type belong? (See Marshall's Microbiology, pp. 306–313.) Suggest from what source each type may come.

B. MICROSCOPIC METHOD

Apparatus. Special capillary pipettes graduated to deliver exactly 0.01 c.c.; clean glass slides; three staining jars; xylol; alcohol, 95%; Loeffler's alkaline methylen blue; stage micrometer; eyepiece micrometer for counting objects in microscopic field; stiff straight needle.

Culture: Fresh milk—same as used in A.

Method. **1.** Draw with ink a figure the size and shape

of an ordinary microscopic slide and on either side and equidistant from the center draw a square whose area is one square centimeter, making the homologous sides of all figures parallel.

2. Place a clean glass slide on the figure.

3. With the capillary pipette, drop over the center of one of the smaller figures exactly 0.01 c.c. of milk directly from the well-shaken sample and with a stiff straight needle spread this drop of milk exactly over the area (one square centimeter) covered by this figure.

4. Make a duplicate smear, placing the drop of milk containing 0.01 c.c. on the slide over the remaining small square.

5. These smears may be dried by the use of gentle heat (e.g., level wooden surface over a steam radiator). Do not allow the smears to become too hot, as this causes them to check, making satisfactory staining impossible.

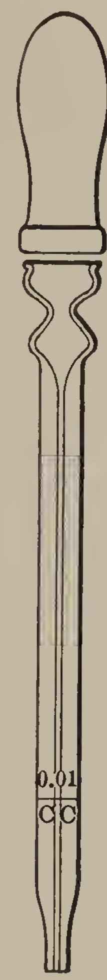


FIG. 58.—Capillary Pipette used in the Microscopic Method for Counting Bacteria in Milk. Note the straight narrow bore and the square tip.

6. As soon as dry, place the slides in a staining jar containing xylol for a short time to remove the fat.

7. Remove the slide from the xylol, absorb the surplus xylol about the edges with filter paper and allow it to dry.

8. Fix the film to the slide by immersing in 95% alcohol.

9. Stain immediately by flooding the smears with Loeffler's methylen blue for two or three minutes.

10. Decolorize to a light blue in 95% alcohol.

11. In counting, use the oil immersion objective. Place the draw tube at some convenient mark so that an even

number of fields of the microscope covers one square centimeter.

To do this, determine the radius of the microscopic field in millimeters with the stage micrometer and calculate its area by the formula πR^2. ($\pi = 3.1416$.)

Then if x = the area of the smear in square millimeters and if 0.01 c.c. of milk is used,

$$\frac{x}{\pi R^2} \times 100 = y.$$

y = the factor necessary to transform the number of bacteria found in one field of the microscope into terms of bacteria per cubic centimeter.

To simplify the calculation, place the draw tube so that y consists of as many ciphers as possible. Convenient factors will be obtained if the length of R be 0.101 mm. or 0.08 mm.

Let z thousand equal the number of fields of the microscope in one square centimeter. Since 0.01 c.c. of milk was taken then each bacterium seen in one field represents $100 \times z$ thousand or z hundred thousand bacteria per cubic centimeter.

12. For careful quantitative work it is necessary to count one hundred fields for each sample, i.e., fifty fields per square. If n = the number of fields counted and m = the total number of bacteria found, the number of bacteria per cubic centimeter is calculated by the following formula:

$$\frac{z \text{ hundred thousand}}{n} \times m = \text{number of bacteria per cubic}$$

centimeter of milk.

In comparatively fresh milk where the bacteria are few, count the whole microscopic field.

An eyepiece micrometer having a circle ruled into quadrants is recommended where large numbers of bacteria are present. The area of the circle is different from that of the

whole microscope field and consequently the factor necessary for computation is different. This factor can be determined by modification of the formula given in **11.**

13. Draw a typical smear from different samples of milk. Indicate the kinds of cells and the number found, also the quality of the milk.

Quality of milk.	Bacteria per field.	No. per c.c.	Tissue cells.	Cell count.
Good..........	None	....	2	800,000 per c.c.
Fair...........	5	2,000,000	1	400,000 per c.c.
Souring normally	200	80,000,000	1	400,000 per c.c.
Poor...........	250	100,000,000	7	2,800,000 per c.c.

14. What types of bacteria are found microscopically? How do these compare with those found on plates as to types and numbers?

What are the advantages and disadvantages of the plating method? Of the microscopic method? For what type of work is each best adapted? What other microscopic methods have been employed as a rapid means of setting bacterial milk standards? How does the Cooledge-Wyant method differ from the numerical count method and what are its advantages?

Of what value are bacteriological milk standards and analyses?

15. Why should the slide be dried rapidly? Give your results in detail and point out any practical applications.

REFERENCES

Brew, James D.: A Comparison of the Microscopical Method and Plate Method of Counting Bacteria in Milk. Bul. 373, N. Y. Agr. Expt. Sta., Geneva, Feb., 1914.

Marshall: Microbiology, Second Edition, pp. 365–368, 403–407.

Löhnis: Laboratory Methods in Agricultural Bacteriology, pp. 62–65.

Savage: The Bacteriological Examination of Food and Water (1914), pp. 85–89, 92, 95–99.
Ward: Pure Milk and the Public Health (1909), pp. 126–128.
Ernst: Milk Hygiene; translated by Mohler and Eichhorn (1914), pp. 24–31.
Frost, W. D.: A Microscopic Test for Pasteurized Milk. Jour. Am. Med. Assn. Vol. LXIV, No. 10, p. 821 (1915)
Cooledge and Wyant: The Keeping Quality of Milk as Judged by the Colorimetric Hydrogen Ion Determination. Jour. Dairy Science, Vol. III, pp. 156–166, 1920.

EXERCISE 2. THE DETERMINATION OF THE BACTERIAL CONTENT OF MILK IN THE UDDER

Apparatus. Several large sterile test tubes; four sterile Petri dishes; 99 c.c. dilution flasks; sterile 1 c.c. pipettes; tubes of sterile litmus milk; four tubes litmus lactose agar.

Method. 1. Wash off the end of the teat very carefully with a solution of mercuric chloride, 1 : 1000; allow it to dry till the surplus solution has disappeared and only sufficient moisture remains to make the cells and any dirt adherent.

2. Secure a sterile cotton-plugged test tube, remove the cotton plug with the little finger and, while holding the mouth of the tube as near the end of the sterilized teat as possible and inclining the tube toward the horizontal position as far as feasible, milk the tube half-full.

By this method obtain from the same teat:

a. One sample of the fore milk,
b. One sample of the middle milk;
c. One sample of the strippings;

Note. For investigational purposes it may be better to employ a sterile milking tube adjusted to a sterile flask. This may easily be prepared. This method, however, is not recommended for student work.

3. Secure one sample from the pail, gathered from the same cow at the same milking.

4. Plate each sample on litmus lactose agar, using the following dilutions and amounts:

1 c.c. of **2***a* diluted 1 : 100 for the plate.
1 c.c. of **2***b* diluted 1 : 10 for the plate.
1 c.c. of **2***c* diluted 1 : 10 for the plate.
1 c.c. of **3** diluted 1 : 100 for the plate.

5. Place the plates at a temperature of 21° C. for seven days.

6. Count the number of colonies in each plate and record the average number in 1 c.c. of milk in each case. Explain any variation in counts.

7. Compare the colonies of plates **2***a*, **2***b*, and **2***c* with **3**. What types predominate?

8. Estimate, so far as possible, the number of colonies of each type, and compare the relative numbers of each species in the different plates.

9. Isolate the species in milk tubes to study their action upon milk. To what group of microorganisms found in milk does each of the species isolated belong? How do you account for the presence of these particular species?

10. In which sample would you expect to find the greatest number of microorganisms? Why?

Why should all samples be taken from the same quarter of the udder?

How do bacteria ordinarily gain entrance to the udder? By what means may bacteria cause infection of the udder?

What is the significance of bacteria in the udder? As to numbers and types?

11. Give your results in full and point out any conclusions and any practical applications possible.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 369–372, 378–386.
Ward: Pure Milk and the Public Health (1909), pp. 1–7, 69, 86–88.

Rosenau: The Milk Question (1912), pp. 71–74.
Russell and Hastings: Outlines of Dairy Bacteriology (1910), pp. 30–35, 75.

EXERCISE 3. TO ILLUSTRATE EXTRANEOUS CONTAMINATION

Apparatus. Forceps; scalpel or spatula; seventeen sterile Petri dishes; three 10 c.c. dilution flasks (for A and B); fifteen sterile 1 c.c. pipettes; fifteen tubes of sterile litmus lactose agar; soap; ordinary towel; two 1 qt. sterile basins; one milk pail; two 1 liter flasks each containing 500 c.c. sterile salt solution; one deep Petri dish; sterile glass rod.

A. SCALES FROM COW'S SKIN (DEAD EPITHELIAL CELLS)

Method. 1. With a sterile spatula scrape from the skin of a cow's udder some scales, such as usually fall into the milk, into a sterile Petri dish.

2. Transfer by means of the sterile spatula some of these to a 75 c.c. Erlenmeyer flask containing 10 c.c. of sterile physiological salt solution.

3. Shake thoroughly, then plate 1 c.c. of this suspension in litmus lactose agar.

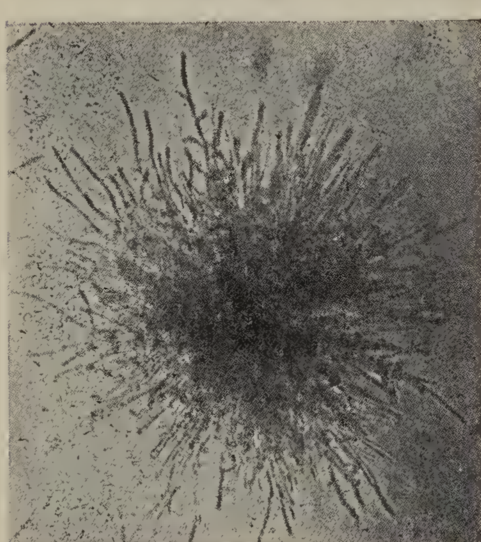


Fig. 59.—*Bact. bulgaricum* colony, x75. (Orig. Northrup.)

B. HAIRS FROM COW

Method. 1. Select two hairs from the back of the cow where the usual or natural cleanliness exists and two from the hip stained with manure. By means of sterile forceps place them in sterile Petri dishes.

2. One of each kind, that is, one from the back and one from the hip, place in 10 c.c. of a sterile salt solution, as under A.

3. Shake thoroughly, then plate 1 c.c. of this suspension in litmus lactose agar.

4. Embed each of the two remaining hairs in the litmus lactose agar after pouring the liquefied agar into a sterile Petri dish. These hairs should be placed in the agar just before solidifying by means of sterile forceps.

C. OTHER SUBSTANCES

Method. Study straw, hay, dung, etc., in a similar manner. Instead of using dilution flasks containing 10 c.c. it will be more desirable to use 100 c.c.

In the case of dung, a particle smaller than the head of a pin should be added to 100 c.c. for suspension, and in case of straw and hay very small segments unless they are very clean.

Note. A sufficient number of such substances should be studied to familiarize the student with the amount of contamination which may take place from these sources.

D. HANDS

Method. 1. Wash the hands in the ordinary manner, rinse them thoroughly, then wipe with an ordinary towel.

2. After this has been done, put 500 c.c. of sterile water in a sterile dish, and rub the hands thoroughly with this water.

3. Plate 1 c.c. of this water in litmus lactose agar.

4. Again rub the hands, before they have been washed and after working for some time, in 500 c.c. of sterile water placed in a sterile dish.

5. Plate 1 c.c. of this water in litmus lactose agar.

6. Compare the numbers (using 1 c.c. as the unit) and kinds of bacteria in the two plates.

E. PAILS

Method. 1. Add to a milk pail washed in the usual manner, 500 c.c. of sterile salt solution, and plate in litmus lactose agar, 1 c.c. of this suspension after it has been moved over the inner surface of the pail.

2. Repeat by using a milk pail heated in steam for ten minutes, or cleansed with boiling water.

3. This same process may be repeated using milk bottles, milk cans, etc.

F. AIR

Method. 1. Determine qualitatively the microflora of the air of the stable before feeding or bedding or before any disturbing, and after feeding or bedding or after any disturbing, by the following methods:

2. Pour the liquefied litmus lactose agar into several Petri dishes, and expose the poured plates for different lengths of time.

3. Expose 10 c.c. of sterile 0.6% salt solution in a deep Petri dish 5 c.c. deep and 9 c.c. in diameter. Try to disintegrate the dust particles by stirring with a sterile glass rod and agitating. Plate 1 c.c. in litmus lactose agar.

4. Quantitative studies of barn air under various conditions may be made according to Exercise 1, Air Microbiology.

5. What advantage has litmus lactose agar over ordinary agar in this exercise?

What types of organisms are met most frequently under A, B, C, D, E and F? How may this occurrence be accounted for?

Which sources furnish the greatest number of organisms? From which sources are the greatest number of microorganisms most likely to enter milk? The most undesirable types? Explain in each case.

What sources of milk contamination have not been dis-

cussed under this exercise? Of what importance is each? What is the simplest method in each case of preventing contamination from the various sources mentioned above?

6. Give your results in full and draw any conclusions and make any practical applications possible.

REFERENCES

SAVAGE: The Bacteriological Examination of Food and Water (1914), pp. 90–91.

ERNST: Milk Hygiene, transl. by Mohler and Eichhorn (1913), pp. 67–102, 125–131, 234–235.

JENSEN: Milk Hygiene, transl. by Pearson (1907), pp. 70–82, 86–127.

MARSHALL: Microbiology, Second Edition, pp. 372–378.

EXERCISE 4. TO INVESTIGATE THE AMOUNT AND KIND OF DIRT IN MILK AND ITS RELATION TO THE MICROBIAL CONTENT OF THE MILK

Apparatus. Six sterile 1 c.c. pipettes; 99 c.c. dilution flasks; six tubes sterile litmus lactose agar; six sterile Petri dishes; sedimentation tubes, 10 c.c. capacity; balance; centrifuge; clean slides; methylen blue, aqueous-alcoholic; physiological salt solution; pneumatic or other type of sediment tester; cotton disks for sediment tester; clean empty milk bottle; one pint bottled milk from each of several miscellaneous sources.

Note. The same sample of milk must be used for A, B and C. Proceed with tests in the order given.

A. DETERMINATION OF MICROBIAL CONTENT OF MILK

Method. 1. Shake the sample in the bottle vigorously.

2. Plate the dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000 in litmus lactose agar.

3. Place the plates at 25° C., and proceed with the microscopic sediment test.

4. Count the plates at the end of five days and estimate

the number of bacteria per cubic centimeter and the relative proportion of acid to other types of colonies.

5. Determine the morphology of the organisms making up the colonies of each type and compare with the findings in the microscopical sediment test.

6. Are all organisms present microscopically? Explain your results and draw conclusions.

B. MICROSCOPIC SEDIMENT TEST

Method. **1.** Mix the milk well and warm about 30 c.c. to 60° to 70° C.

2. Place 10 c.c. of this well-mixed, warmed milk into each of two sedimentation tubes.

3. Place one tube on each of the scale pans and balance by adding more milk to the lighter tube. The tubes must be equal in weight or they will throw the centrifuge "off center."

4. Centrifuge in a machine designed for this purpose for five minutes, till a more or less considerable compact sediment separates out.

5. Pour or pipette off the milk above the sediment.

6. Fill the tubes with physiological salt solution and mix the sediment well throughout the dilution fluid with a platinum needle.

7. Balance the tubes and centrifuge again.

8. Pour or pipette off the physiological salt solution.

9. With a small platinum loop, obtain a small amount of the sediment and make a smear on a clean slide.

10. Stain with aqueous-alcoholic methylen blue.

11. Determine the proportions of bacteria and leucocytes in ten fields. Also note the presence of bacteria in clumps and foreign matter.

The presence of many leucocytes and streptococci associated together is generally indicative of an inflamed condition of the udder, as in mastitis (garget). On the other

hand, sometimes the milk from normal udders may show a considerable quantity of leucocytes in the sediment.

C. MACROSCOPIC SEDIMENT TEST

Method. 1. Put a cotton disk in place in the pneumatic sediment tester, heat the sediment tester and clean

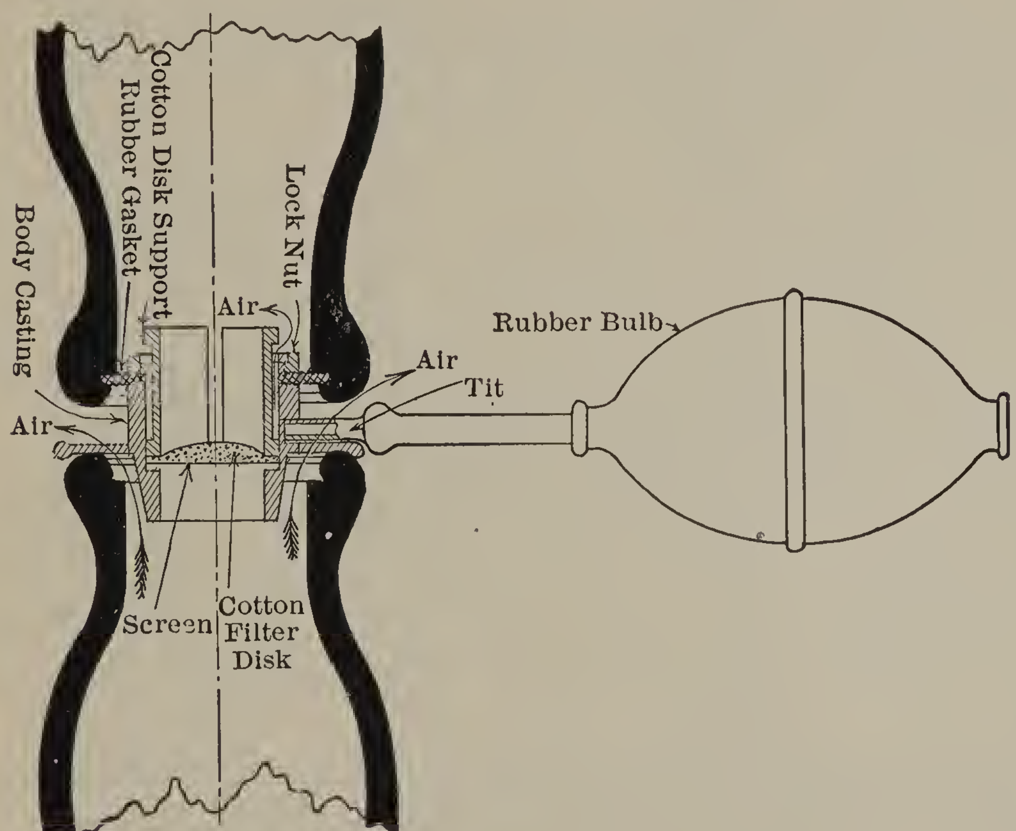


FIG. 60.—Wizard Sediment Tester for Milk.

empty milk bottle in steam thirty minutes, and allow to cool.

2. Attach the sediment tester to the top of the milk bottle containing the sample of milk, using "aseptic" precautions, and invert the whole apparatus over the mouth of the sterile empty milk bottle.

3. Pump the contents of the upper bottle into the lower bottle by means of the rubber bulb. The milk is forced through the cotton disk and leaves its larger particles of insoluble dirt on the cotton.

4. Note the quality of the milk tested by this method.

What is the difference between the microscopic sediment test, and the macroscopic sediment test?

5. What does the presence of visible dirt on the cotton indicate? Is this sediment test an argument for straining milk before it goes to the consumer? Is it an argument for running milk through a milk clarifier before putting it on the market?

6. Immediately after straining, plate the milk in litmus lactose agar, using dilutions 1 : 100, 1 : 10,000 and 1 : 1,000,000 as before.

7. Incubate the plates for five days at 25° C. and count, estimating total average number and proportions of types as in A.

8. Compare the counts with those of A, also the proportions of the various types.

Note. This method was formerly used for obtaining an estimate microscopically of the numbers of bacteria in milk. It presents difficulties, however, which lead to many technical errors and therefore it cannot be relied upon to give uniform results. The method is valuable, however, for determining something of the sanitary quality of the milk.

Did straining have any effect on the numbers of organisms present in the milk? What effect may it have? Is this beneficial to the milk as a commercial product?

9. In what way may the microscopic sediment test explain the results obtained by plating milk before and after straining?

10. Make, stain and examine smears from the upper surface of the cotton disk. What is the nature microscopically of the material retained by the cotton? How does this smear compare qualitatively with that from the centrifuged sample?

11. What is the nature of the dirt ordinarily found in milk? How may its presence be eliminated?

12. Give all results in full and draw any conclusions permissible. Point out any practical applications.

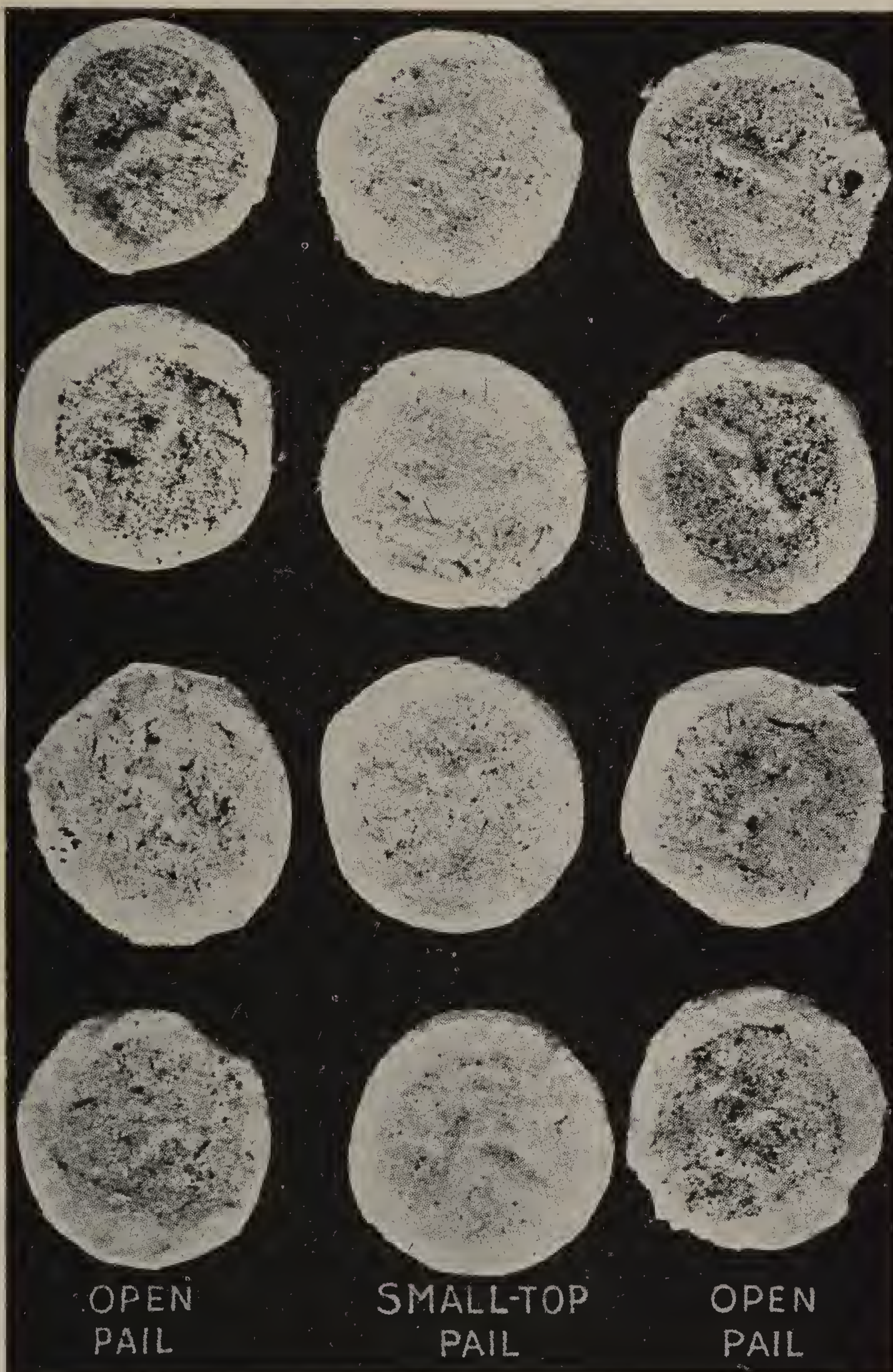


Fig. 61.—Cotton Disks Prepared by the Use of the Wizard Sediment Tester. (Circ. 41, Wisc. Expt. Sta.)

REFERENCES

Löhnis: Laboratory Methods in Agricultural Bacteriology, pp. 63–65.
Jensen: Milk Hygiene, transl. by Pearson (1907), pp. 126–127.
Marshall: Microbiology, Second Edition, pp. 399–403.
Rosneau: The Milk Question (1912), pp. 55–88.
Ernst: Milk Hygiene, transl. by Mohler and Eichhorn (1914), p. 182.

EXERCISE 5. TO DETERMINE THE INFLUENCE OF TEMPERATURE UPON THE KEEPING QUALITY OF MILK; FRESHLY DRAWN MILK COMPARED WITH ORDINARY MARKET MILK

One of the most important considerations in the production of milk, either for factory use or for town or city supply, is the temperature at which the milk is maintained. The beneficial effects of scrupulous cleanliness in the production of milk will be largely counteracted unless the milk is cooled immediately after drawn and maintained at a temperature too low for development of the bacteria present.

Apparatus. Three sterile 1 liter Erlenmeyer flasks; one sterile 2 liter Erlenmeyer flask; twenty-four sterile Petri dishes; sterile 10 c.c. pipettes; sterile 1 c.c. pipette graduated to 0.1 c.c.; twenty-four tubes of litmus lactose agar; 90 c.c. and 99 c.c. dilution flasks; ice and salt for preparing freezing mixture; fresh milk and bottled milk.

Method. 1. In a sterile 2 liter Erlenmeyer flask place about 1½ liters of milk from a can of milk immediately after it has been filled by the milkers.

Note. This exercise is to be repeated, for purposes ofcomparison, using three pint bottles of milk all obtained at one time from the same milkman. In this case the first plating is to be made from each separate bottle.

2. Record the temperature. Plate from the sample immediately in litmus lactose agar, making dilutions of 1 : 100 and 1 : 500. Determine the acidity of the sample, using a sterile 5 c.c. pipette to obtain the sample.

Note. Portions for acidity determination and plating should be removed with sterile pipettes in all instances.

3. Transfer the sample "aseptically" into the three 1 liter flasks, placing an equal portion as nearly as possible in each flask. Label the flasks *A*, *B*, *C*.

4. Cool flask *A* in a freezing mixture to 10° C., and set away in refrigerator to maintain the low temperature.

5. Cool flask *B* in a freezing mixture to 10° C., then place it at a constant temperature of 21° C.

6. Place flask *C* at a constant temperature of 21° C.

7. At the end of twenty-four hours determine and record the acidity of each of the three portions of the original sample.

8. Plate in litmus lactose agar, using the following dilutions:

> Flask *A*, 1 : 100 and 1 : 1,000.
> Flasks *B* and *C*, 1 : 10,000 and 1 ; 1,000,000.

9. At the end of another twenty-four hours repeat the titrations and platings with all flasks, using the following dilutions:

> Flask *A*, 1 : 10,000 and 1 : 1,000,000.
> Flasks *B* and *C*, 1 : 1,000,000 and 1 : 100,000,000.

10. At the end of five days determine and record the acidity of the milk in all flasks. Plate from flask *A* only, using dilutions of 1 : 1,000,000 and 1 : 100,000,000.

11. All plates should be held at 21° C. for a period of five days before counting.

12. Compare the relative kinds and numbers of colonies in plates from the three flasks. Note also the time of curding and the nature of the curd formed in each case.

13. Compile the results of the investigation in tabulated form. Plot bacterial count and acidity curves.

14. From the results obtained, what conclusions would

you draw as to the influence of cooling upon the keeping quality of milk?

How does the age and original quality of the milk effect its keeping qualities when subjected to different temperature conditions?

How does cooling milk and keeping it cool compare with merely cooling and then allowing the milk to acquire the temperature of the room? What is the explanation of the action occurring?

What is the purpose of cooling the milk as soon as it comes from the cow? What different methods are used? What are some of the disadvantages of the different methods used for cooling?

What bacterial action takes place in the refrigerator milk? Is the germicidal action of milk sufficiently important to recommend a change in the general practice of cooling milk?

15. Give your results in detail and point out any practical applications or conclusions.

REFERENCES

Ward: Pure Milk and the Public Health (1909), pp. 15–16, 24–25, 37, 121.

Russell and Hastings: Outlines of Dairy Bacteriology, pp. 54–56.

Ernst: Milk Hygiene, transl. by Mohler and Eichhorn (1914), pp. 148–149, 156.

Marshall: Microbiology, Second Edition, pp. 390, 391.

EXERCISE 6. A STUDY OF THE PASTEURIZATION OF MILK OR CREAM BY LABORATORY METHODS

Apparatus. Water bath; test-tube rack of metal to fit water bath; sterile, large tubes selected for uniformity in diameter (2 cm.); sterile Petri dishes; sterile 1 c.c. pipettes, graduated to 0.1 c.c.; sterile litmus lactose agar tubes.

Method. 1. Secure milk or cream, about 125 c.c. to be used for tubing and pasteurizing.

Note. If time permits, it is desirable to test pasteurization upon:

a. Fresh milk or cream.

b. Milk or cream which has stood for twenty-four hours but is still sweet.

c. Milk or cream which has reached an acidity of about 22°.

d. Milk or cream from different sources, supposedly having different bacterial contents.

2. Tube the sample or samples of milk or cream, pouring 10 c.c. into each tube, filling fifteen tubes for each sample.

Note. Only one sample should be pasteurized at a time.

3. Prepare one tube from each sample of milk or cream for the introduction of the thermometer. By so doing, the conditions being practically identical, the temperature will be easily read and controlled.

4. After the tubes are prepared mark tubes in duplicate as follows: 50°, 60°, 70°, 80°, 90° and 100°, leaving two unmarked as controls.

5. Place them in the rack so that the marks on the tubes may be easily recognized, and insert the rack in the water-bath.

6. Pour water into the water-bath until the height of the water corresponds to the height of the milk in the tubes.

7. Put aside two tubes of milk or cream from each sample, one to be employed for comparative check-observation, and the other for check-plating against those which will be subjected to pasteurization.

8. Apply heat to the water-bath.

9. At 50°, 60°, 70°, 80°, 90° and 100° C., remove two tubes of each sample of milk or cream undergoing pasteurization and place in cold water.

10. Employ one of the tubes thus removed for plating and the other place at a temperature of 25° to 28° C. along with the previous check-observation tube (**7**).

11. Make two plates in litmus lactose agar from the tube held for check-plating (**7**) and from one of the two tubes removed at each of the temperatures designated above. The remaining tube is to be left undisturbed and placed at 25° C., to observe macroscopical changes.

Dilutions for plating:

Fresh milk, unpasteurized, 1 : 10 and 1 : 100.

Milk twenty-four hours old, but sweet, unpasteurized, 1 : 10,000 and 1 : 1,000,000.

Milk with an acidity of 22°, unpasteurized, 1 : 100,000 and 1 : 10,000,000.

Milk pasteurized at 50° C. (fresh) 1 : 10 and 1 : 100.

Milk pasteurized at 50° C. (old) 1 : 10,000 and 1 : 1,000,000.

Milk pasteurized at 60° C., 1 : 10 and 1 : 100.

Milk pasteurized above 60° C., 1 : 10.

12. Keep the plates at 25° C. for seven days, counting colonies at the end of this time.

13. Determine the character of the microorganisms left after pasteurization with those before pasteurization as to the relative number of each kind, to the fermentation of milk or cream, to spore formation, and to resistance. Which microorganisms have succumbed to pasteurization at different temperatures and which were able to withstand it?

14. Record the results obtained from the study of plates and cultures made from colonies.

15. Record your observations from day to day of macroscopical changes in the pasteurized and unpasteurized control tubes. Does pasteurization destroy organisms that are favorable, or detrimental to the milk? What influence does pasteurization have upon the keeping quality of milk?

16. What influence do the following factors have upon the efficiency of pasteurization: the age of milk? acidity? degree of temperature to which milk is subjected? dura-

tion of pasteurization temperatures? presence or absence of air? pressure, whether atmospheric or greater? viscosity or other changes in milk or cream?

What changes are accomplished by pasteurization? Why is milk pasteurized? Is this end always accomplished in commercial plants?

What different methods are used commercially for the pasteurization of milk? What are the advantages and disadvantages of each method? Why?

At what stage in the process of production should milk be pasteurized to accomplish the desired results? Must the after-treatment of pasteurized milk be any different from that of unpasteurized milk?

Do you think that milk should be pasteurized before it reaches the consumer?

Does pasteurization affect the digestibility of milk? What are the limitations of pasteurization as applied to milk?

17. What effect does pasteurization have on enzymes? On vitamines? Give your results in full and any conclusions that may be drawn.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 391–393.

Rosneau: The Milk Question (1912), pp. 16, 37, 76, 105, 112, 120, 128, 132, 138, 161, 185–230, 294.

Ward: Pure Milk and the Public Health (1909), pp. 71, 73, 74, 114–125.

EXERCISE 7. DETERMINATION OF THE NUMBER AND TYPES OF BACTERIA IN BUTTER

Apparatus. Three tubes litmus lactose agar; litmus milk tubes; fresh butter; sterile dilution flasks; three sterile Petri dishes; sterile 1 c.c. volumetric (bulb) pipettes.

Method. **1.** Melt a small composite sample of butter in a

test tube at the *lowest possible temperature* (between 40° and 45° C.). Mix well.

2. Using a warm pipette, transfer 1 c.c. of the well-mixed melted butter to 99 c.c. of sterile (warm) salt solution. Free the pipette from fat by filling it with the dilution water several times. Use warm (50° C.) pipettes and dilution flasks throughout so that the butter will not stick to the pipettes and may be readily emulsified.

3. Plate in litmus lactose agar, using dilutions 1 : 1,000, 1 : 100,000 and 1 : 1,000,000.

Note. These dilutions may have to be changed. Look up the average number of bacteria in the type of butter you are using and make dilutions accordingly.

4. Incubate the plates at 25° C.

5. Weigh 1 c.c. of well-mixed melted butter and record the weight in grams.

6. Examine the plates after three to five days for acid and other types of colonies.

7. Count and record the number of bacteria per cubic centimeter, also the types. Note the action of each type on litmus milk.

8. Estimate the number of bacteria per gram.

9. What is the melting-point of butter? Are bacteria ordinarily killed at this temperature?

What kinds of microorganisms are found in fresh butter from ripened cream? In old butter? In fresh oleomargarine? In renovated butter? In canned butter?

Do bacteria increase or decrease in butter kept in storage? What other methods of making a bacteriological examination of butter may be employed?

Are microorganisms in any way responsible for the flavors of butter? Explain.

What pathogenic organisms may gain entrance to butter?

What is the avenue of entrance? How long can bacteria

exist in butter? How do bacterial numbers and types compare with those of fresh milk? of ripened cream?

10. Give your data and conclusions in full and point out any practical applications.

REFERENCES

RUSSELL and HASTINGS: Practical Dairy Bacteriology, pp. 95, 97.

LÖHNIS: Laboratory Methods in Agricultural Bacteriology, pp. 81–82.

MARSHALL: Microbiology, Second Edition, pp. 408–419

SAYER, RAHN and FARRAND: Keeping Qualities of Butter, I. General Studies, Tech. Bul. I (1908), Mich. Agrl. Expt. Sta.

RAHN, BROWN and SMITH: Keeping Qualities of Butter, II and III. Tech. Bul. 2 (1909), Mich. Expt. Sta.

WELLS, LEVI: Renovated Butter: Its Origin and History. Yearbook of Dept. of Agr. for 1905, pp. 393–398.

ROGERS, L. A.: Studies Upon the Keeping Quality of Butter, I. Canned Butter. Bul. 57, B. A. I., U. S. Dept. of Agr. (1904), pp. 6–8, 22–23.

TANNER, F. W.: Bacteriology and Mycology of Foods, pp. 423–428.

GUTHRIE, E. S.: The Book of Butter, pp. 160–180.

EXERCISE 8. TO DETERMINE THE NUMBER AND TYPES OF MICROORGANISMS IN CHEESE

Apparatus. Cheddar cheese; cheese trier (sterile) if an uncut cheese is to be sampled; mortar, with pestle; two knives, sterile; quartz sand; sterile filter papers about 6 and 8 cm. square; one dilution flask containing 95 c.c. of 0.85% salt solution; dilution flasks, containing 90 and 99 c.c. sterile 0.85% salt solution; sterile 1 c.c. and 10 c.c. pipettes; three tubes litmus lactose agar; three sterile Petri dishes; sterile litmus milk tubes.

Method. **1.** Sterilize in the hot-air oven, 10 gms. of sand in a mortar with a pestle.

2. Using a red-hot knife blade, sear a portion of the surface of the cheese.

3. To weigh the cheese "aseptically," place the smaller sterile filter paper upon the larger on the balance pan.

4. With a sterile knife remove the inner portion of the seared surface and obtain and weigh out 5 gms. of cheese, using aseptic precautions.

5. Transfer the cheese to the sterile mortar and grind up well.

6. Transfer the cheese and sand mixture with sterile knife or spatula to the 95 c.c. dilution flask. Shake well to free the sand from the cheese.

Directions for making dilutions. In transferring with a pipette a portion of the first suspension to other dilution flasks the sample should be taken immediately after shaking before the sand has settled. Settling may be avoided by holding the pipette in a horizontal position until ready to deliver the contents. The grinding material should be fine enough to avoid clogging the pipette.

7. Make and plate the following dilutions in litmus lactose agar: 1 : 100,000; 1 : 10,000,000 and 1 : 1,000,000,000, if the cheese has been made recently.

If the cheese is not perfectly fresh, use dilutions 1 : 100,000; 1 : 1,000,000 and 1 : 10,000,000. Lower dilutions may be necessary if the cheese has been stored for some time.

8. Incubate the plates at 25° C. for three to five days.

9. Count and estimate the number of microorganisms per gram of cheese. What types predominate? Why?

10. Transfer the different types to litmus milk and note the action after several days. Which of these types, as determined from the action on litmus milk, may have a prominent part to play in the ripening of the cheese? Why?

12. Do microorganisms play any part in the formation of the flavor of cheddar cheese? Other cheeses?

How does the cheese analyzed compare with the butter analyzed as to numbers and types of microorganisms?

What qualitative tests are made for milk used for cheese making? What is the principle and application of these tests?

What cheese "abnormalities" may be caused by microorganisms? During what stages in the process of making cheese may these occur?

What pathogenic organisms have been found in cheese? What is known of their longevity in this medium?

13. State your results and conclusions in full and point out any practical applications.

REFERENCES

Löhnis: Laboratory Methods in Agricultural Bacteriology, pp. 83–88.

Russell and Hastings: Experimental Dairy Bacteriology, pp. 103–109.

Marshall: Microbiology, Second Edition, pp. 387, 420–437.

Conn: Practical Dairy Bacteriology (1907), pp. 223–259.

Savage: The Bacteriological Examination of Food and Water (1914), pp. 118–119.

Decker: Cheese Making (1905), pp. 48–51, 63, 66, 69, 75, 88, 108–110.

Vanslyke and Publow: The Science and Practice of Cheese-making (1912), pp. 115–135, 285–312, 371–378.

Tanner, F. W.: Bacteriology and Mycology of Foods, pp. 428–432.

EXERCISE 9. A COMPARISON OF THE BACTERIAL CONTENT OF SWEETENED AND UNSWEETENED CONDENSED MILKS

Apparatus. Six sterile Petri dishes; six tubes of litmus lactose agar; 99 c.c. dilution flasks; two 95 c.c. dilution flasks; tubes of sterile litmus milk; two sterile 5 c.c. pipettes with large aperture for delivery; can-opener.

Culture. Unopened can of sweetened condensed milk; unopened can of unsweetened condensed milk (contents guaranteed to be sterile).

Method. **1.** Sterilize the can-opener in the flame.

2. Thoroughly cleanse the outside of the unopened cans of condensed milk and then submerge in boiling water for five or ten minutes.

3. Remove the cans from the water, being careful in handling them not to contaminate the upper surface of the cans.

4. With the sterile can-opener make an opening in the can only large enough to admit the introduction of a 5 c.c. pipette.

Note. Only one can is to be opened at a time to avoid contamination.

5. With a sterile pipette obtain a 5 c.c. sample from the can just opened and transfer to a 95 c.c. dilution flask.

Note. As the condensed milk is very viscous and adheres to the sides of the pipette, after delivering the 5 c.c. into the dilution flask blow out the remainder into the sink or other suitable place, then replace in the dilution flask and wash out the adhering fluid by drawing the diluting fluid up into the pipette several times. The use of a 5 c.c. volumetric pipette having a large aperture for delivery would lessen the possibilities of contamination.

6. This resulting dilution is a 1 : 20 dilution of the condensed milk, or a 1 : 40 dilution of the original milk (if the directions on the can give a dilution of 1 : 1 for producing a milk of original composition).

7. Plate the following dilutions of the condensed milk in litmus lactose agar: 1 : 20, 1 : 2000 and 1 : 20,000. Place plates at 25° C.

8. Examine and count at the end of five days.

9. Record the numbers and types of organisms developing on the plates. Are any acid colonies present? Determine the morphology of the acid colonies.

10. Transfer each type of colony to a tube of sterile litmus milk and observe action from day to day. Are the types which are present desirable? Is *Bact. lactis acidi* present? Any organisms of the *B. coli* type? Are pathogenic bacteria apt to be present?

11. To what factors are due the keeping qualities of each type of condensed milk?

What care should be taken of opened cans of milk of either type? Of the milk after it has been diluted according to directions?

In what other forms is concentrated milk sold? What

factors are responsible for the keeping quality of each of these latter types?

12. Give your results and conclusions in detail.

REFERENCES

MARSHALL: Microbiology, Second Edition, pp. 438–441.
SAVAGE: The Bacteriological Examination of Food and Water (1914), pp. 111–113.
SADTLER: Industrial Organic Chemistry (1912), pp. 281, 288.
TANNER, F. W.: Bacteriology and Mycology of Foods, pp. 433, 434.

EXERCISE 10. TO DETERMINE THE NUMBER AND TYPES OF MICROORGANISMS IN ICE CREAM

Apparatus. Litmus lactose agar shake; three tubes sterile litmus lactose agar; three sterile Petri dishes; sterile 1 c.c. pipettes; sterile dilution flasks; sterile wide-mouthed glass-stoppered bottle; sterile butter trier; sterile knife.

Culture. From ice cream.

Method. 1. Remove the (frozen) ice cream sample from the container by means of the sterile butter trier.

2. With the sterile knife discard the upper portion of the sample and place in the sterile wide-mouthed bottle.

Note. Pack the sample in ice if it cannot be examined at once.

3. To examine, allow the ice cream to melt quickly by placing it at about 37° C. and then treat as a milk sample.

4. Plate on litmus lactose agar, using the following dilutions: 1 : 10,000, 1 : 1,000,000 and 1 : 100,000,000 and incubate plates at 37° C.

5. Add a large quantity (25 c.c. to 50 c.c.) to the melted agar shake and incubate at 37° C. Examine in twenty-four to forty-eight hours for acid and gas. Is *B. coli* present?

6. Count plates at the end of three days and estimate the total number of bacteria present per cubic centimeter, also the number of acid colonies and of any other predominant type.

7. Transfer predominant types to litmus milk tubes and note action, also note rapidity with which each type produces changes in the litmus milk. What may these results signify?

8. Make a microscopic count, using the method in Exercise 1, Dairy Microbiology. How do microscopic and plate counts compare?

9. Look up references for ascertaining bacteriological standards for ice creams. What is the quality of the ice cream you analyzed as compared with the maximum bacterial limit? What do you think this limit should be?

10. From what diverse sources do bacteria enter ice cream?

What is their significance in this product?

What relation may some of the common practices of ice-cream makers have to the bacterial content of milk?

What effect does storage have upon the number of bacteria in properly hardened ice cream?

What significance has a pure ice-cream supply in relation to public health?

11. Give results and conclusions in detail.

REFERENCES

Savage: The Bacteriological Examination of Food and Water (1914), pp. 119–121.

Marshall: Microbiology, Second Edition, pp. 447–449.

Wiley, H. W.: Ice cream, Hygienic Lab. Bul. 56. Milk and its Relation to the Public Health (1909), pp. 251–311.

Hammer, B. W.: Bacteria and Ice Cream, Bul. 134 (1912), Iowa Agr. Expt. Sta.

Mortensen, M., and Gordon, J.: Lacto: a New and Healthful Frozen Dairy Product. Bul. 119 (1911), Iowa Agr. Expt. Sta.

Washburn, R. M.: Principles and Practice of Ice-cream Making. Bul. 155. Vermont Agr. Expt. Sta., pp. 9–10, 34–46, 53–54, 64–66.

Bolduan: Food Poisoning (1909), pp. 84–90.

Ayers, S. H., and Johnson, Jr., W. T.: A Bacteriological Study of Retail Ice Cream. Bul. 303, U. S. Dept. Agr., 1915.

Tanner, F. W.: Bacteriology and Mycology of Foods, pp. 434–439.

Fabian, F. W.: A Score Card for City Ice Cream Plants. Journal of Dairy Science, Vol. III, No. 3, May, 1920.

PLANT MICROBIOLOGY

EXERCISE 1. TO DEMONSTRATE THAT PLANTS ARE SUBJECT TO MICROBIAL DISEASES: INFECTION OF CERTAIN SPECIES OF VEGETABLES HAVING JUICY ROOTS, LEAVES, FRUITS, ETC., WITH B. CAROTOVORUS

Apparatus. Tubes of sterile 2% saccharose broth; tubes of sterile agar; sterile water; sterile Petri dishes; three sterile deep culture dishes; sterile filter paper; sterile knife; sterile forceps; mercuric chloride, 1 : 500; juicy vegetables.

Culture. *B. carotovorus* (culture of high physiological efficiency).

Method. **1.** The root of the carrot, turnip, rutabaga, the cucumber or radish; the cotyledons of immature pea seedlings, petioles of cabbage seedlings, potatoes, etc., may be used for this exercise. For what other plants is *B. carotovorous* pathogenic?

2. Thoroughly wash the root, or vegetable to be inoculated. Two or three vegetables of one kind should be employed.

3. Disinfect a spot about 2 cm. in diameter with 1 : 500 mercuric chloride and rinse with sterile water to get rid of disinfectant. Drain off excess moisture on sterile filter paper, handling vegetable with sterile forceps.

4. Puncture the disinfected area on one vegetable with the sterile stiff needle for control and place in a sterile deep culture dish.

5. Obtaining some of the culture of *B. carotovorus* on the sterile needle, puncture the remaining vegetables in the center of the disinfected area and place vegetables in a sterile deep culture dish at 20° to 25° C.

B. carotovorus is a wound parasite which invades the intercellular spaces, dissolving the middle lamellæ and

portions of the inner lamellæ, thereby establishing a condition which is known as soft rot.

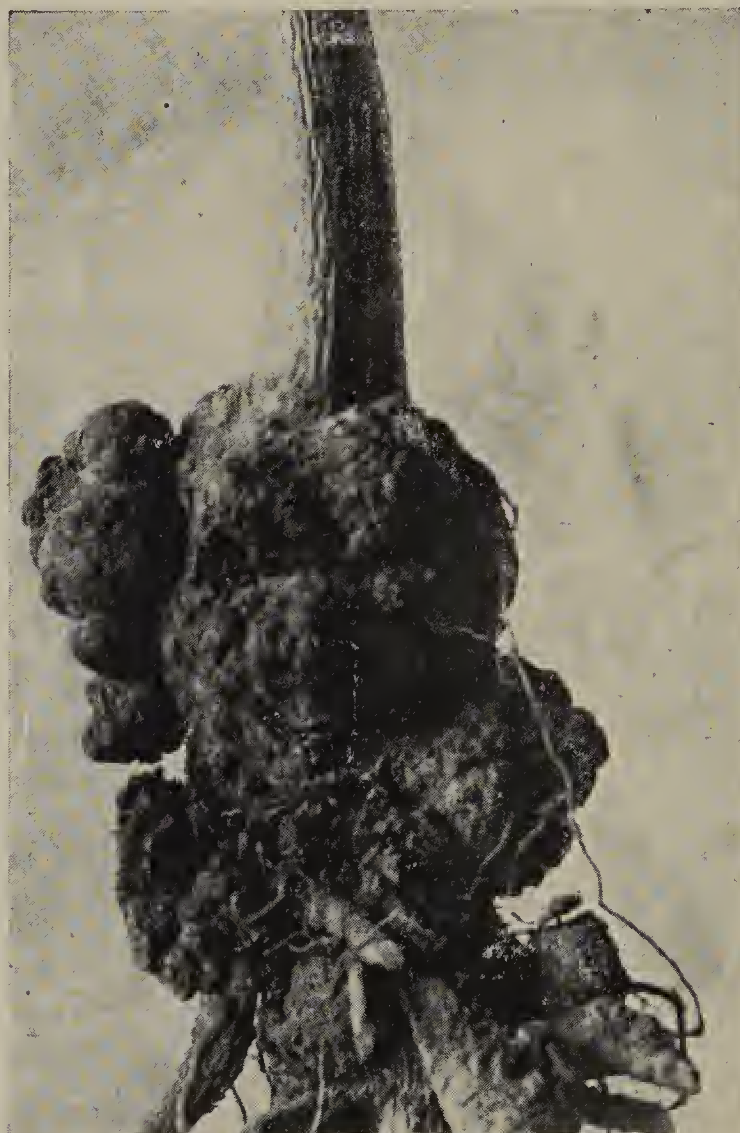


Fig. 62.—Crown Gall Produced by *Bact. tumefaciens*. (Orig.)

6. Examine in twenty-four hours for evidence of action of *B. carotovorus*. This should be easily distinguishable in three days.

7. Isolate the causal organism and determine its morphology and cultural characteristics. Compare with pure culture and with description given in Marshall's Microbiology, p. 624.

Is the organism newly isolated, capable of producing infection? Make inoculations from one, two, three and

four-day old newly isolated cultures to sterile living vegetable tissue to determine this. Is there any difference in the infectivity of a one-day old and a three- or four-day old culture?

8. What is known of methods of control of this disease?

9. Grow four giant colonies of *B. carotovorus* on ordinary agar, one in each Petri dish and allow them to develop until nearly 1 cm. in diameter.

10. Under sterile conditions, remove slices of fresh carrot, beet and rutabaga or turnip roots and potato and place in sterile Petri dishes. (Slices should be at least 3 to 4 cm. in diameter.)

11. With a sterile scalpel make a circular incision 0.5 cm. from the edge of the colony through the layer of agar in the Petri dish.

12. Remove this colony intact to the surface of one of the slices of vegetable and replace cover of Petri dish.

13. Repeat, removing a colony to the slice of each of the different vegetables.

14. Examine in twenty-four hours for evidences of soft rot, and note progress of softening from day to day. What is demonstrated by this phenomenon? Are all vegetables attacked?

15. What parts of the plant does *B. carotovorus* attack? What chemical constituents of these parts are decomposed through the agency of their action?

What are the main features of difference in the mechanism of action of the various types of bacterial plant diseases?

Give an example of a disease illustrating each.

How is the progress of infection effected in these various types? What organisms produce a disease of similar type in other vegetables and plants?

What is known of immunity in the plant kingdom?

What methods of control are employed with different types of plant diseases? How are methods of control influenced by the type of disease?

Note. This exercise may be made more interesting and instructive if combined with histological methods.

Plates illustrating the invasion of root tissues by *B. carotovorus* are found in Smith's Bacteria in Relation to Plant Diseases, Vol. I, pp. 56, 103.

16. State in full your results and conclusions.

REFERENCES

Smith, Erwin F.: Bacteria in Relation to Plant Diseases, Vol. I, pp. 5, 6, 65, 86, 103. Vol. II, pp. 51–52, 65, 81–88, 96, 292. Vol. III.

Marshall: Microbiology, Second Edition, pp. 588–631.

Jones, L. R.: A soft rot of carrot and other vegetables, pp. 299, 13th Rept. of Vt. Expt. Sta. (1901). Also in Cent. f. Bakt. II, Bd. 14, pp. 369–377.

Jones, L. R.: Pectinase, the cytolytic enzyme produced by *B. carotovorus* and certain other soft-rot organisms. Tech. Bul. 11, N. Y. Agr. Expt. Sta. (1909).

ANIMAL DISEASES AND IMMUNITY

EXERCISE 1. ANIMAL INOCULATION IN BACTERIOLOGY FOR DETERMINATION OF THE IDENTITY OF A MICROORGANISM, ITS PATHOGENICITY OR VIRULENCE, OR FOR PRODUCTION OF IMMUNITY

Apparatus. Experimental animals: rabbits; guinea pigs; white rats; white mice, etc.; scalpels; scissors; forceps; razor; syringe; trephine; sterile dishes; anesthetic; disinfectant; cotton.

Culture. Pure culture or infected material.

I. INTRODUCTION

1. Avoid the use of animals where the employment of other means answers the purpose equally well.

2. Unless other factors prevent, always use the most susceptible and least expensive animals.

II. PREPARATION OF ANIMAL *

Method. 1. (*a*) Examine carefully each animal before subjecting it to experimentation.

(*b*) Use no animal already showing symptoms of illness or general lack of vigor.

(*c*) Record the weight and temperature of each animal.

2. (*a*) Administer an anesthetic (general or local as indicated) whenever the operation is very painful or tedious or where perfect immobility of the parts is required.

Note. For local anesthesia a 2% solution of cocaine hydrochloride may be made by dissolving 0.1 gm. of cocaine hydrochloride in 5 c.c. of sterile water. Instill a few drops into the conjunctival sac or inject 1 to 5 c.c. into the subcutaneous tissues near the seat of operation. For general anesthesia 10 to 30 c.c. of a 5% solution of chloral hydrate may be injected per rectum, or ether or chloroform may be inhaled.

* The instructor must arrange for experiments that must be started early in order to be completed before the term closes.

Ether is probably safer in the hands of a novice. It may be administered by saturating cotton placed in a paper cone which is kept over the animal's nose. Care should be exercised to replenish the supply of the anesthetic on the cotton as fast as it volatilizes and not to force the anesthetizing too fast. Injury to the integument about the nose may be avoided by rubbing on vaseline before beginning the operation. The tissues should not be cut until anesthesia is complete.

(*b*) Choose a site for operation where the results will not interfere with the animal's locomotion or normal functions.

(*c*) Use sharp, sterile instruments.

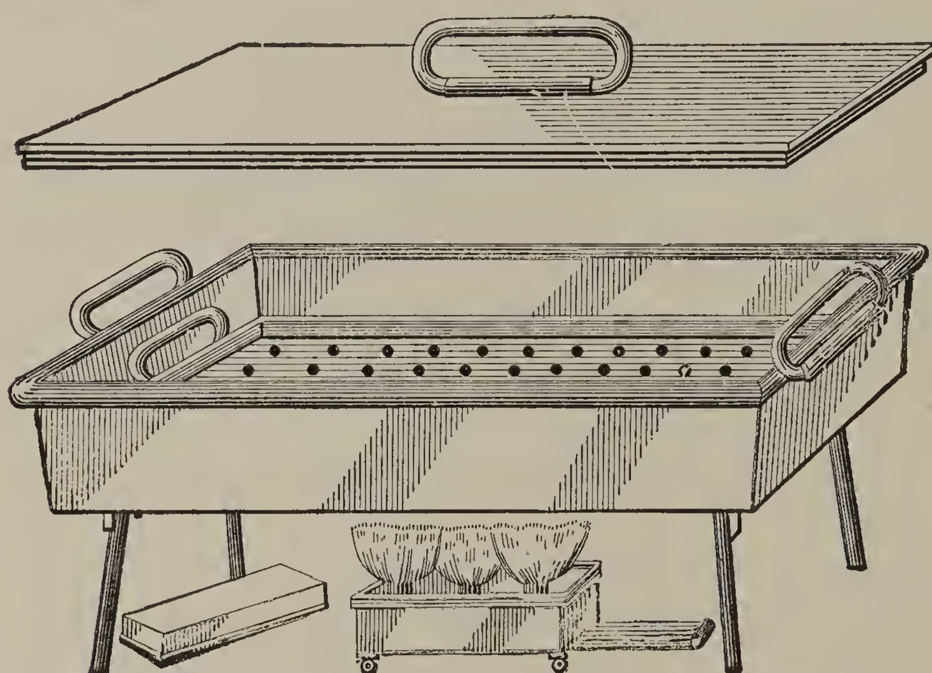


FIG. 63.—Tray for Sterilizing Surgical Instruments.

Note. Methods for holding different animals for different forms of operations vary. An assistant is usually required to hold the animal, where an anesthetic is not administered, and where an anesthetic is used it is usually better to have an assistant administer it, although this is not necessary. (For various devices for holding experimental animals see text-book: Eyre, Bacteriological Technic, 2d Ed. (1913), pp. 349–352.)

3. Remove the hair with scissors or clippers from the field of operation and shave the surface. Wash the skin and disinfect it with 2% liquor cresolis compositus (U. S. P.). Wash off the disinfectant with alcohol and allow the

alcohol to evaporate. The animal is now ready for the operation.

Note. It is understood that a 2% solution of liquor cresolis compositus (U. S. P.) shall be used wherever a disinfectant solution is indicated unless otherwise stated.

III. METHODS

Where the exact nature of the inoculum is unknown, the experimenter will be guided, as to what method to select, by his judgment, influenced by experience with other inocula in animal experimentation. The method most adaptable in the case of each specific microorganism will be indicated in the treatment of that organism.

1. *Cutaneous.* Rub the inoculum on the shaved and disinfected skin or make several parallel, superficial incisions and rub the inoculum into the scarifications with a sterile scalpel. See that no disinfectant remains on the skin before operating.

2. *Subcutaneous.* I. (*a*) Pick up the skin with the thumb and forefinger of the left hand and insert the needle through one side of the fold of skin thus made.

Note. The point of the needle should not enter the skin on the other side of the fold, but should lie in the subcutaneous tissue.

(*b*) Release the skin and inject the material.

(*c*) Place the finger moistened with the disinfectant over the point where the needle enters the skin and remove the needle.

II. (*a*) For solid material that will not pass through a hypodermic needle, make a short incision through the skin parallel to the horizontal plane of the body.

(*b*) With a sterile probe separate the skin from the underlying tissues on the lower side of the cutaneous incision, making a small pocket in the subcutaneous tissue.

(*c*) With fine-pointed sterile forceps insert the inoculum

into this pocket. Further treatment should not be necessary.

3. *Intramuscular.* I. Plunge the needle deeply into the muscles, preferably on the inside of the thigh.

II. Inject the material slowly with steady pressure if the volume is great.

4. *Intravenous.* I. Inject the liquid into the ear vein

Fig. 64.—One Method of Injecting Hog-cholera Serum. (Orig.)

of the rabbit (and other animals if possible) or jugular vein where accessible. The injection should be in the direction of the circulation.

II. The femoral vein may be used where other veins are not readily entered with the needle. Use general anesthesia. Make an incision on the inside of the thigh over the femoral space. Separate the iliacus, pectineus and sartorius muscles. The femoral vein and artery are laid bare. After inoculation disinfect and suture the skin.

Note. Solid substances, larger than leucocytes, and air bubbles should not be injected into the vascular system. Fatal emboli may result. Return of blood through the needle indicates that the vein has been entered. If swelling occurs at point of inoculation the inoculum is entering the subcutaneous tissue. Try again.

5. *Intraabdominal or intraperitoneal.* I. The site for the operation is the center of the angle formed by the last rib, transverse processes of the lumbar vertebræ, and the external angle of the ilium.

(*a*) Plunge the needle or trocar and canula through the abdominal wall with one thrust.

Note. When the parietal peritoneum is punctured the sudden disappearance of resistance to the entrance of the needle is noticed. The intestines will not be entered if pressure on the needle stops at this point.

(*b*) Inject the material and remove the needle, placing the thumb and finger on each side of the needle and pressing gently on the skin during the removal so as to prevent separating the skin and underlying layers of tissue.

II. Infectious material or cultures in a sterile collodium capsule may be introduced into the abdominal cavity by performing laparotomy. (General anesthesia is desired.)

6. *Intraorbital.* Always perform under local anesthesia. (2% cocaine hydrochloride.)

I. Steady the eye with fixation forceps.

II. Pierce the cornea near to its periphery with a fine needle. The needle should incline with the point outward so that, upon entering the anterior chamber of the eye, the iris will not be damaged.

III. Inject the material.

7. *Subdural.* Operate under general anesthesia.

I. Make a longitudinal incision through the skin at one side of the sagittal suture. Hold back the skin and subcutaneous tissue with tenacula.

II. Make a crucial incision through the periosteum and push back the four corners.

III. Expose the dura mater by removing a small button of the parietal bone (0.5 cm. in diameter) with a trephine.

IV. Inject the inoculum immediately beneath the dura mater.

V. Replace the periosteum and suture the skin.

VI. Disinfect.

8. *Intrapulmonary.* I. Pull the animal's front leg forward.

Fig. 65.—Another Method of Injecting Hog-cholera Serum. (Orig.)

II. Plunge the needle through the fifth or sixth intercostal space into the lung tissue.

III. Slowly inject the contents of the syringe.

Note. In large animals material may be injected into the trachea between the tracheal rings.

9. *Ingestion.* If possible mix the infectious material with the animal's food.

Note. See that the animal eats all that is intended to be eaten.

Introduce the infectious material into a gelatin capsule and force the animal to swallow it; or give the material as a drench where advisable.

Note. Fasting the animal before introducing unpalatable material into the food may be helpful in increasing the amount eaten. The chemical reaction of the stomach contents as governed by physiological activity will influence results.

IV. CARE OF INOCULATED ANIMALS

1. Watch each animal closely and take temperatures as the case demands.

2. Treat each animal as a case of infectious disease in quarantine.

Note. Whatever clinical, diagnostic or sanitary measures necessary in that given disease may be employed as seen fit.

3. When the animal is removed from the cage for the last time, carefully destroy all refuse in the cage and disinfect thoroughly.

4. Give all results, observations and conclusions in detail.

REFERENCES

Stitt: Practical Bacteriology, Blood Work and Parasitology, Fifth Edition, pp. 62, 101, and 128.

Moore and Fitch: Bacteriology and Diagnosis, pp. 114, 118–120, 124, 125, 130, 133.

Eyre: Bacteriological Technic, pp. 332–369.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 53–64.

EXERCISE 2. THE ISOLATION OF PATHOGENIC BACTERIA FROM FLUIDS AND TISSUES OF DEAD ANIMALS

Apparatus. Disinfectant; scalpel; scissors; forceps; bone forceps; ten sterile pipettes; 10 c.c. sterile pipettes; 250 c.c. flask containing glass beads, sterile; sterile Esmarch dishes; spatula; platinum loop; special media.

Method. 1. Disinfect the skin.

2. Remove the spleen, kidney, lymph glands, and any other diseased tissue, to sterile Esmarch dishes, using sterile instruments.

3. Collect samples of pericardial and pleuritic fluids, blood, urine and bile with sterile pipettes and place these in small sterile flasks. Collect at least 25 c.c. of blood in a sterile flask containing glass beads for defibrinating.

4. Remove the organs collected to the laboratory and make cultures as follows:

5. Sear the surface of the organ with a spatula heated to a white heat.

6. Tear the seared surface with forceps, sterilized in flame.

7. With a sterile platinum loop, make transfers to agar slants, shake cultures, and plates for isolation into pure cultures.

8. Repeat **7**, using any body fluids collected.

Note. The different diseases require special procedures and media for successful results. Attention will be called to these variations at the proper places.

REFERENCES

Moore and Fitch: Bacteriology and Diagnosis, pp. 95–96.
Moore: Principles of Microbiology, pp. 156–162, 237–258.
Eyre: Bacteriological Technic, pp. 248–258.

EXERCISE 3. A STUDY OF BACT. ANTHRACIS

Note. *Bact. anthracis* is the cause of anthrax, a disease very fatal to man and certain domestic animals. Great care should be taken while working with it.

Apparatus. Six tubes of agar; three tubes of potato; three tubes of milk; tube of gelatin; slides and stains; autopsy instruments.

Culture. *Bact. anthracis.*

Method. **1.** Inoculate three tubes each of agar, potato, and milk and one tube of gelatin with *Bact. anthracis.*

2. Incubate one tube of each at 20° C., one at 37° C., and one at 42° C. (Study and record the effect of these temperatures upon the growth and spore formation of the organism.)

3. Make cover-glass preparations and stain with methylen blue, fuchsin and Gram's stain. Stain for spores (Anjeszky's method).

4. Transfer a small quantity of the agar culture to 4 or 5 c.c. of sterile physiological salt solution and inject 0.25 c.c. subcutaneously into a guinea pig. Make daily observations and an autopsy of the animal at death.

5. Make cultures on agar slants, and smear preparations from the blood, liver, spleen and kidney after the autopsy.

6. Fix the smears in the flame, stain with methylen blue or fuchsin. After twenty-four and forty-eight hours examine the cultures microscopically.

7. State your results and conclusions in full.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 86, 199, 208, 233, 503, 562, 570, 705, 707, 750–754.

Jordan: General Bacteriology, Fifth Edition, pp. 233–245.

Besson: Practical Bacteriology, Microbiology and Serum Therapy, transl. by Hutchens (1913), pp. 517–535.

Kolmer: Infection Immunity and Specific Therapy (1917), pp. 700–702.

Zinsser: Infection and Resistance (1914), pp. 15, 18, 53, 64, 296.

EXERCISE 4. THE PREPARATION OF TUEERCULIN

Apparatus. Two 500 c.c. Erlenmeyer flasks; glycerinated veal broth; evaporating dish; 0.5% phenol salt solution; Berkefeld filter; heavy filter paper; 20 c.c. homeopathic vials; sealing wax.

Culture. *Bact. tuberculosis* (specially adapted for tuberculin).

Method. **1.** Place about 200 c.c. of glycerinated veal

bouillon in each of two 500 c.c. Erlenmeyer flasks and sterilize for twenty minutes each day for three consecutive days.

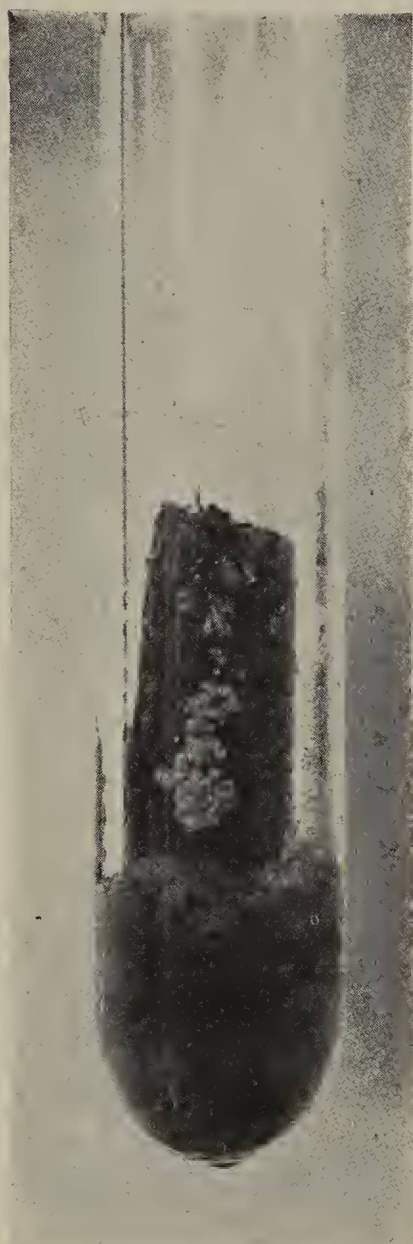


Fig. 66.—*Bact. tuberculosis* (avian), on Banana. (Orig. Himmelberger.)

2. From a culture of the tubercle bacterium furnished, inoculate each flask of veal broth. In making the inoculations care should be taken to place the inoculum on the surface and to avoid agitation after inoculation. Seal the flasks with paraffin and place in the incubator at a temperature of 37° C.

3. Allow the cultures to grow four weeks after the surface is covered, then shake well, place in a steam sterilizer and subject to steam for two and a half hours.

4. Filter through a filter paper to remove most of the bacterial growth.

5. Evaporate to one-tenth its original volume over a water bath at a temperature of 60° C.

6. To one volume of the concentrated tuberculin add seven volumes of sterile physiological salt solution containing 0.5% phenol or tricresol and then filter through a Berkefeld filter.

7. Place the product in 20 c.c. homeopathic vials and seal with wax. Label the vials and place in a cool dark room.

REFERENCES

MARSHALL: Microbiology, pp. 485, 487.
MOORE: Principles of Microbiology, pp. 251–253.

FIG. 67.—Glycerin Veal-broth Cultures of *Bact. tuberculosis* (Human), for Tuberculin, about Eight Weeks Old. (Orig. Keck.)

BESSON: Practical Bacteriology, Microbiology and Serum Therapy, pp. 289–345.
MOORE: Bovine Tuberculosis.
KOLMER: Infection, Immunity and Specific Therapy (1917), pp. 716–730.
ZINSSER: Infection and Resistance (1914), pp. 355–357, 438–442.

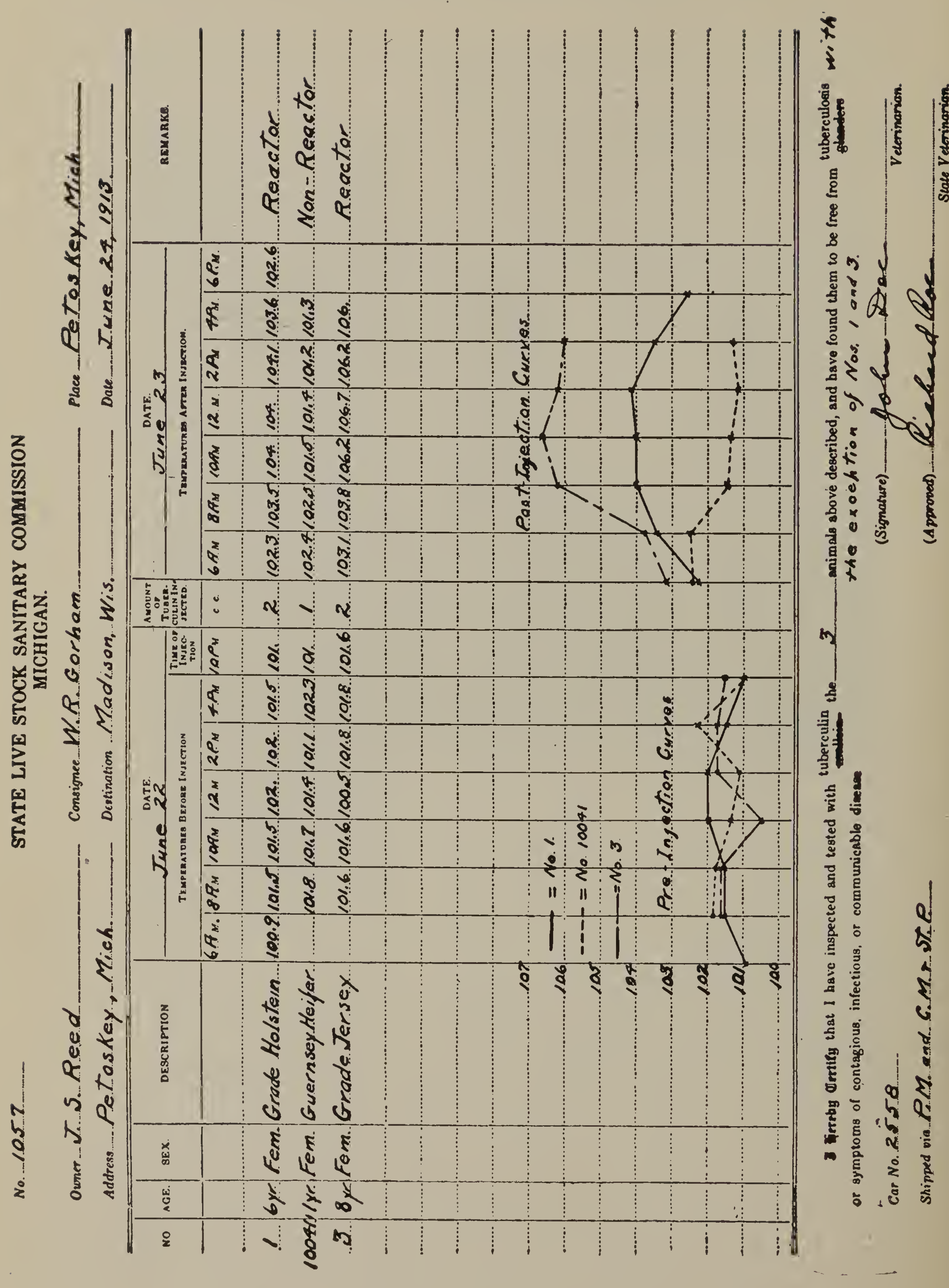


FIG. 68.—Tuberculin Test Chart.

EXERCISE 5. THE PREPARATION OF BLACK-LEG VACCINE

Apparatus. Sterile mortar and pestle; sterile cheesecloth; two sterile glass plates; sterile water; sterile homeopathic vials.

Culture. Diseased muscle of calf affected with blackleg.

Method. **1.** Place a piece of diseased muscle from a calf affected with black leg (this will be furnished by the instructor) in a sterile mortar, add a small quantity of water and triturate completely with a sterile pestle.

2. Squeeze the pulp through a piece of sterile cheese cloth, spread the filtrate in a thin layer over a sterile glass plate or saucer and dry at a temperature of 35° to 37° C. in an atmosphere free from contamination.

3. Mix approximately one part by volume of the dried virus with two parts of water, triturate until the mixture is converted into a semi-solid homogeneous mass and spread in a thin layer over a glass plate or saucer.

4. Heat in an oven to a temperature of 100° to 104° C. for a period of seven hours.

5. One centigram of the attenuated virus mixed with a small quantity of water is a dose for a calf. Place the product in sterile vials, ten doses to the vial, place a cork stopper in each vial and seal.

REFERENCES

Norgaard, V. A., and Mohler, J. R.: Black leg, its Nature, Cause and Prevention, B. A. I. Circular No. 31, revised (1911).

Marshall: Microbiology, Second Edition, pp. 565, 566.

Kolmer: Infection, Immunity and Specific Therapy (1917), p. 702.

EXERCISE 6. THE PREPARATION OF TETANUS TOXIN

Apparatus. Dextrose broth; two 100 c.c. Erlenmeyer flasks; paraffin oil; 5% phenol; Berkefeld filter.

Culture. *B. tetani.*

Method. **1.** Place 50 c.c. of dextrose bouillon in each of two 100 c.c. Erlenmeyer flasks, plug and boil gently two or three minutes over a flame.

2. Cover the bouillon with a layer of paraffin oil about 5 mm. deep and heat in the autoclav.

3. After cooling, inoculate the bouillon with *B. tetani* and incubate about two weeks.

4. Examine the culture microscopically to determine the absence of contamination, add sufficient 5% phenol to make a 0.5% solution and filter through a Berkefeld filter.

5. Incubate about 1 c.c. of the filtrate in 10 c.c. of dextrose broth under anaerobic condition, for forty-eight hours to make sure that the filtrate is sterile.

6. The filtrate, if sterile, is to be used in immunizing a rabbit for the production of antitoxin.

Note. A fairly potent toxin will kill a guinea pig in a 0.001 c.c. dose. The toxin in solution is very unstable and should be kept in a tightly stoppered bottle in a cool dark place. It may be kept for several months by precipitating with a saturated solution of ammonium sulphate and drying in vacuo over sulphuric acid.

REFERENCES

Marshall: Microbiology, Second Edition, p. 579.

Besson: Practical Bacteriology, Microbiology and Serum Therapy (1913), pp. 536–548.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 772–781.

Zinsser: Infection and Resistance (1914), pp. 41, 107, 131–133.

EXERCISE 7. THE PREPARATION OF TETANUS ANTITOXIN

Note. In the preparation of tetanus antitoxin for therapeutic purposes, healthy horses are used. For the first injection of toxin a small fraction of a cubic centimeter is given subcutaneously. The increase in the size of the dose and the frequency of injection depend upon the condition of the animal, but the quantity injected is gradually increased until the animal is able to stand 300 to 400 c.c. of toxin at one injection.

For laboratory purposes, the rabbit may be used to furnish the antitoxin.

The following method suggested by Roux and Vaillard produces a satisfactory antitoxin for laboratory study.

Apparatus. Tetanus toxin; Gram's iodin solution; rabbits; 20 c.c. syringe; disinfectant; anesthetic; operating tray; 50 c.c. sterile glass cylinder.

Method. 1. Give the first five or six injections subcutaneously, subsequent ones may be given intraperitoneally.

1st day, 3 c.c. of toxin mixed with 1 c.c. of Gram's iodin solution.

5th day, 5 c.c. of toxin mixed with 2 c.c. of Gram's iodin solution.

9th day, 12 c.c. of toxin mixed with 3 c.c. of Gram's iodin solution.

16th day, 5 c.c. of undiluted toxin.

23d day, 10 c.c. of undiluted toxin.

30th day, 15 c.c. of undiluted toxin.

The quantity may be gradually increased until the rabbit is getting 100 c.c. of undiluted toxin.

2. After the 6th injection has been given, wait a period of ten days and bleed the rabbit aseptically. This is accomplished as follows:

(*a*) Secure the rabbit in a dorsal position on an operating tray and anesthetize with ether.

(*b*) Expose an area about 3 cm. square over the inferior

thoracic wall, in the region of the apex of the heart, shave and clean with alcohol.

(*c*) Insert a sterile needle attached to a sterile 20 c.c. syringe, through the thoracic wall into the heart and slowly draw the plunger out.

If only a small quantity of serum is desired for testing, the animal may be saved for subsequent bleedings.

(*d*) Place the blood in a sterile container, allow to clot and draw off the serum for standardization.

REFERENCES

Marshall: Microbiology, Second Edition, pp. 575, 576, 579 and 580.

Besson: Practical Bacteriology, Microbiology and Serum Therapy (1913), pp. 544–548.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 242–249, 772–781.

Zinsser: Infection and Resistance (1914), p. 463.

EXERCISE 8. A DEMONSTRATION OF THE AGGLUTINATION TEST

Note. There are two methods of applying the agglutination test: First, by combining the suspect's serum in varying amounts with a suspension of the specific organism and incubating eighteen to thirty-six hours; the results are then read with the unaided eye. Second, the serum may be combined in varying dilutions with a suspension of the specific organism, and hanging drop preparations made and examined microscopically. If agglutinins are present, clumping of the organisms will occur in a few minutes. With either method, controls, containing the organism but normal serum, should be prepared for comparative purposes.

Apparatus. Four agar slants; test-tube rack for small test tubes; twelve small test tubes; antiserum; physiological salt solution; 1 c.c. pipettes, graduated to 0.01 c.c.; 5 c.c. pipettes; cover-glasses; concave slide.

Culture. *B. typhosus* or *B. choleræ suis*.

Method. *Macroscopic Test.* **1.** *Antigen.* This is a suspension of the specific organism obtained from a twenty-four to forty-eight hour agar culture in physiological salt

solution. Only a sufficient quantity of the growth to give a slight cloudiness to the salt solution in a small test tube should be used.

Note on Antigen. Where a series of agglutination tests are to be made at intervals, the antigen should be standardized so that the same concentration will be used for each test. Great care should be used in preparing the antigen to avoid clumps in suspension. In some cases thoroughly shaking in a shaking machine will afford a satisfactory antigen, in others it must be filtered through a filter paper.

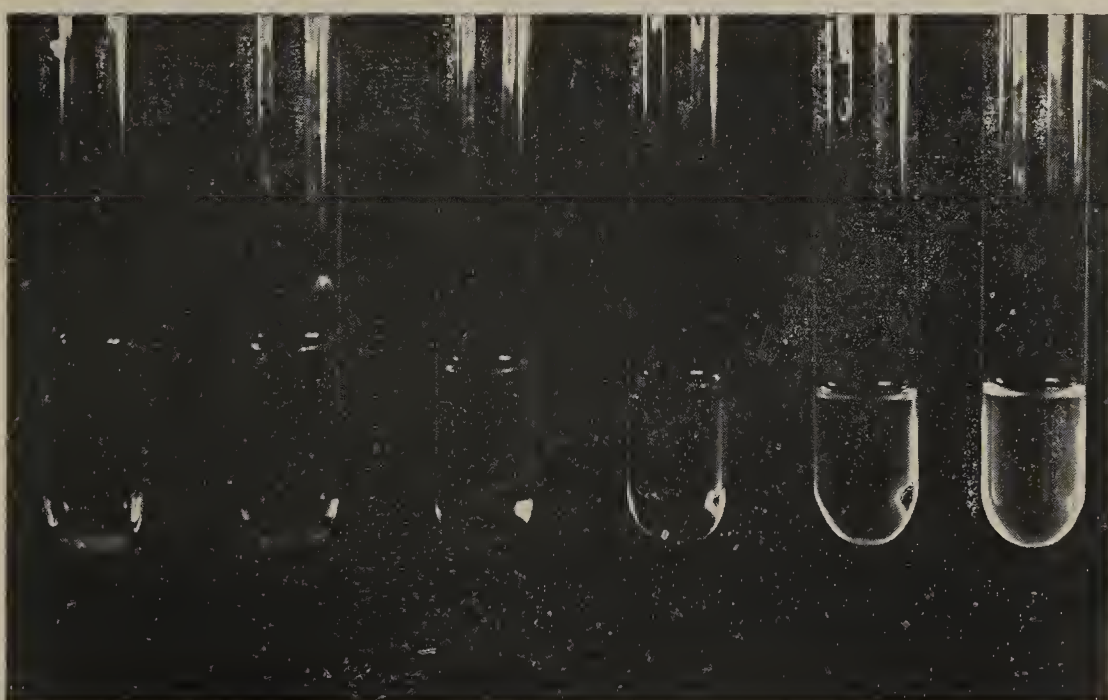


Fig. 69.—Macroscopic Agglutination of *B. choleræ suis* by Dorset-McBryde-Niles Serum. From left to right tubes show, first, complete agglutination, heavy sediment, clear supernatant liquid; in each succeeding tube the sediment becomes less, the turbidity greater, the tube at the right showing uniform cloudiness and no sediment, no agglutination. (Orig. Giltner.)

2. The antiserum may consist of immune serum—a rabbit immunized to the typhoid bacillus may be used to furnish the serum—, or hog cholera serum or virus may be used with *B. typhosus* and *B. choleræ suis* respectively.

3. The following table shows the various combinations of serum, antigen and salt solution to give definite dilutions. Physiological salt solution should be used in diluting the serum.

Tube.	Antigen.	Serum Diluted 1–10.	Salt Solution.	Dilution.
	c.c.	c.c.	c.c.	
1	4	1.0	0.0	1–50
2	4	0.5	0.5	1–100
3	4	0.2	0.8	1–250
4	4	0.1	0.9	1–500
		Serum diluted 1–100.		
5	4	0.5	0.5	1–1000
6	4	0.2	0.8	1–2500
7	4	0.1	0.9	1–5000

4. Shake all tubes well and incubate at 37° C. for twenty-four hours and record the results.

Microscopic Test. **1.** If this test is carried out during the same period with the macroscopic test, a small loopful of the dilution from any tube may be transferred to a clean cover-glass placed on a hanging drop slide and the edges sealed with vaselin or oil. It may then be examined with a microscope.

2. If done independently of the macroscopic test, prepare the suspension of organisms in one test tube and the dilutions of serum in others.

Mix a loopful of the diluted serum with a loopful of the antigen on a clean cover-glass, mount on a concave slide and observe with a microscope for a period of thirty minutes to one hour.

3. Give results and any conclusions in detail.

REFERENCES

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 279–313, and 919.

Marshall: Microbiology, Second Edition, pp. 585, 586, 714–717.

McFarland: Pathogenic Bacteria and Protozoa, 7th Ed. (1912), pp. 149–152.

Giltner: Studies of Agglutination Reactions in Hog Cholera during the Process of Serum Production (Preliminary) Tech. Bul. 3 (1909), Mich. Agr. Expt. Sta.

Giltner: Same title Tech. Bul. 8 (1911), Mich. Agr. Expt. Sta.

Zinsser: Infection and Resistance (1914), pp. 218–247.

EXERCISE 9. A STUDY OF FILTERABLE VIRUSES

Apparatus. Physiological salt solution; Chamberland filter with water-suction or air pump and pressure gage; sterile flasks; clinical thermometer; syringe; flasks of bouillon, 50 c.c. in each; autopsy set.

Culture. Hog cholera virus (blood of hog sick with cholera).

Method. **1.** *Preparation of the Filter.* If the filter has been used once clean it by:

(*a*) First rinsing with cold water under the tap.

(*b*) Force about 1 liter of cold distilled water through it.

(*c*) Then a solution consisting of 1 gm. $KMnO_4$ and 6.5 gms. HCl in 1000 gms. water.

(*d*) Next, 1000 c.c. of a solution of 1% oxalic acid.

(*e*) Boiling water is then forced through the filter until the liquid which runs through is free from acid.

(*f*) Lastly, cold distilled water must be forced through the filter.

Thus treated, any organic residue is destroyed and the filter is as good as new.

This method of purification must always be used *immediately after* using a filter. Filter candles *must not* be left twenty-four hours without cleaning.

A new filter may be prepared for use by forcing through it a large quantity of boiling distilled water and finally cold distilled water.

The amount of liquid necessary to force through the filter for cleaning varies with the size of the filter. The ordinary 8 inch filter should receive the full amount (1000 c.c.) of each solution and distilled water for efficient purification.

Filters are best sterilized by being set up ready to use and autoclaved. (See Fig. 71 for one method.)

2. Procure some hog cholera virus and after diluting it

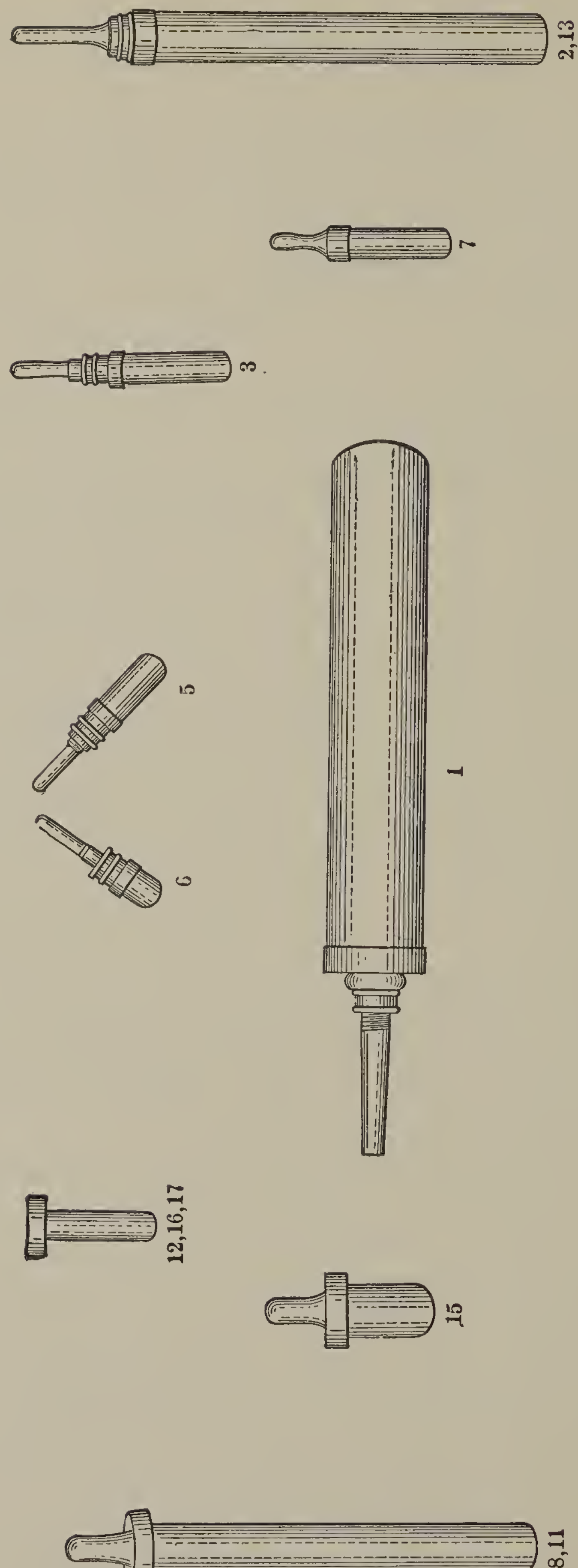


FIG. 70.—Various Types of Bacterial Filters.

with equal parts of physiological salt solution, pass it through a clean, sterile, Chamberland filter at a pressure not to exceed one atmosphere and during a time not to exceed one hour.

3. Make sub-cultures of the filtrate by introducing 1 c.c. into each of several flasks of bouillon containing 50 c.c. each. Take every precaution against contamination. Also make microscopical preparations.

4. If no growth results under 2 inject 2 c.c. into the muscles of a 50 lb. pig. Make daily observations of the pig and record the temperature each day.

5. When undoubted symptoms of hog cholera have developed, kill the pig and make a careful autopsy. Save the blood in a sterile jar.

6. Repeat the experiment, using blood procured in **4** as virus.

7. By repeated filtrations and injecting into susceptible hogs, it may be proven that a living microorganism, incapable of producing visible growth *in vitro*, passes through the filter and develops in the body of the pig.

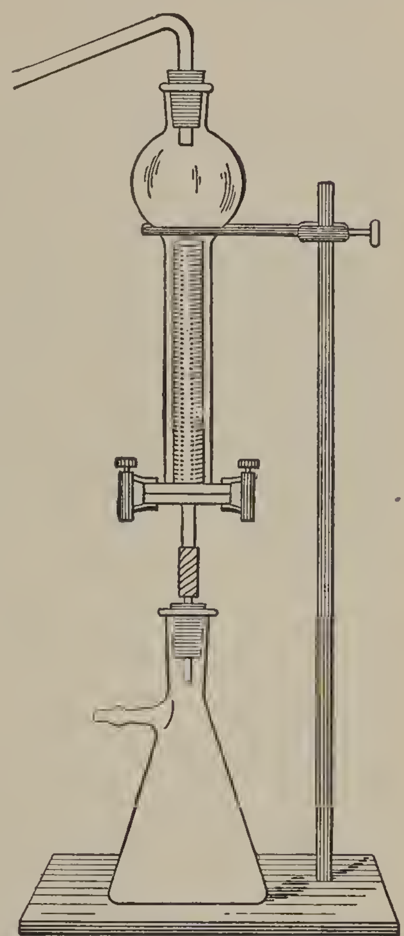


Fig. 71.—Pasteur-Chamberland Filter Adjusted for Filtration by Suction.

8. State your results and conclusions in full.

REFERENCES

Dorset, McBryde and Niles: Further Experiments Concerning the Production of Immunity from Hog Cholera, Bul. 102, B. A. I., U. S. Dept. Agr.

McBryde: Filtration Experiments with *B. choleræ suis*, Bul. 113, B. A. I., U. S. Dept. Agr.

Giltner: What is the Antigen Responsible for the Production of Antibodies in Hog Cholera Serum? Tech. Bul. 13, Mich. Agr. Expt. Sta.
Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 77–78.

EXERCISE 10. THE PREPARATION OF BACTERINS OR BACTERIAL VACCINES

Apparatus. Scalpel; scissors; forceps; sterile tubes and Esmarch dishes; sterile swabs; sterile physiological salt solution; 50% alcohol; disinfectant; four agar slants; six agar tubes for plating; six sterile Petri dishes.

Culture. Infected material or specific cultures to be furnished by the instructor.

A. AUTOGENOUS BACTERINS

Method. In the preparation of an autogenous bacterin, it is first necessary to isolate the microorganism causing the disease. This is accomplished as follows:

1. If there are any unopened abscesses, open one with a sterile scalpel after first disinfecting the field with 2% compound solution of cresol and washing with 50% alcohol. Collect some of the pus on a sterile swab and suspend in sterile physiological salt solution.

2. If the abscess is already opened, using a sterile curette, obtain some of the diseased tissue at the bottom of the abscess and macerate this in sterile physiological salt solution.

3. Pour agar plates from this salt solution suspension, using at least six, plated in series of two.

4. Incubate the plates and after twenty-four hours make observations on the number and type of colonies. After forty-eight hours make transfers to agar slants of the most numerous type of colony. Colonies should be studied under low power of the microscope.

5. Grow three or four cultures of the organism on slanted

agar. Make a morphological study of the organism. After twenty-four hours wash off the growth from each tube with 3 c.c. of sterile saline solution.

6. Put the suspension all in one container, reserving 1 c.c. to be used in standardization.

Note. In the hemocytometer method for standardizing bacterins it is desirable to use a special hemocytometer with a counting chamber 0.02 mm. deep provided with a special cover-glass for counting bacteria, but if this is not accessible, an ordinary hemocytometer and cover-glass as used for blood counting may be used. If the latter, a 4 mm. objective must be used for counting.

Using the diluting pipette of the blood counting apparatus the suspension of bacteria is diluted to the desired dilution with Collison's fluid made as follows:

Hydrochloric acid, 2 cc.

Mercuric chloride 1–500, 100 cc.

Acid fuchsin, 1% aqueous solution—enough to color to a deep cherry red.

Filter before using.

The bacterial suspension is allowed to remain in the pipette eight to ten minutes to stain, then thoroughly agitated by rotating the pipette and the first few drops from the arm of the pipette discarded. The mount is then prepared and the slide placed on the stage of microscope which has been previously leveled, and the count made. The count and calculations are made as for blood counting.

7. Heat in a water bath at 60° C. for one hour. This is usually sufficient to kill the bacteria, unless they are spore producers.

8. To test the sterility of the suspension after heating, with a sterile loop make an agar streak and incubate for twenty-four hours. If growth is obtained the culture must be heated again.

B. STOCK BACTERINS

The procedure in the preparation of a stock bacterin is the same as in the preparation of an autogenous bacterin, except that the organisms used are from cultures kept in stock for that purpose.

C. POLYVALENT BACTERINS

Polyvalent bacterins are those which are prepared from several species of bacteria, e.g., *M.* (*Staph.*) *albus*, *M.* (*Staph.*) *aureus*, *Strep. pyogenes*, etc.

The suspension of each must be prepared and standard-

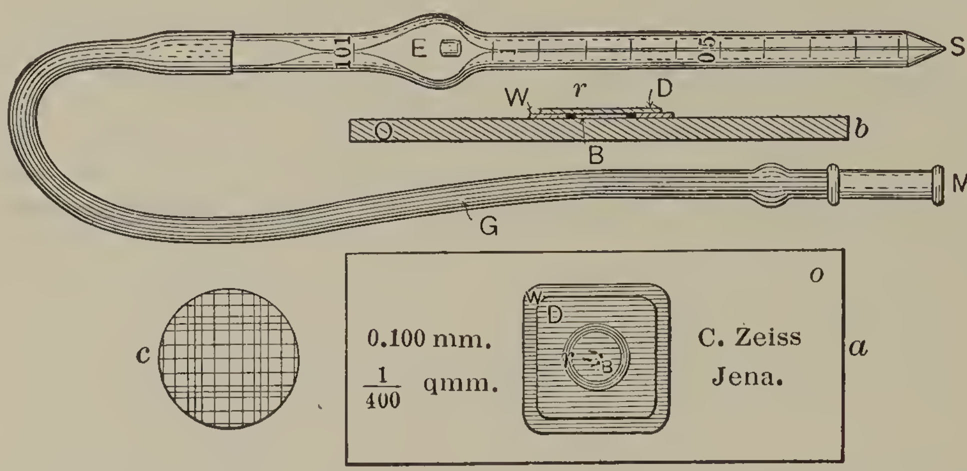


Fig. 72.—Blood-counting Apparatus for Use in Standardizing Bacterial Vaccines.

ized separately, and then the emulsions of all mixed. In this way, it is possible to have a known number of each species in the resulting product.

REFERENCES

Fitch C. P.: A Review of the Principal Methods Used to Standardize Bacterins (Bacterial Vaccines). Report of the N. Y. State Vet. College for the year 1913–1914, pp. 207–219.

McCampbell: Laboratory Methods for the Experimental Study of Immunity, pp. 186, 188.

Stitt: Practical Bacteriology, Blood Work and Parasitology, (1918), pp. 134, 212–215.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 654, 732.

Zinsser: Infection and Resistance (1917), pp. 217, 635, 214.

EXERCISE 11. TO DEMONSTRATE PHAGOCYTOSIS AND SOME INFLUENCING AGENTS

Apparatus. Six or more sterile capillary pipettes; one or more rubber nipples, six clean slides; 10 c.c. syringe with needle; eight bouillon tubes; several agar slants; sterile citrated salt solution and physiological salt solution; Wright's or Jenner's stain; sterile thick-walled bottle containing glass beads; sterile corks or rubber stoppers to fit bottle; sterile 25% solution of peptone or aleuronat solution; guinea pigs or rabbits; 19 gauge aspirating needle; several sterile centrifuge tubes; chloroform or ether; sterile scalpels, forceps, and scissors; McFarland's nephelometer; two funnels with cotton filter wrapped up and sterilized; three or more small sterile test tubes; filed sterile Berkefeld or Chamberland filter.

Culture. *B. typhosus* and *Staph. pyogenes aureus* or *albus.*

Method A. **1.** Inoculate broth tubes with *B. typhosus* and make daily transfers (to broth) for about four days.

2. Inject three guinea pigs of the same weight from the last twenty-four hour cultures, giving 1 c.c. to pig No. 1, 4 c.c. to pig No. 2, and 8 c.c. to pig No. 3. If pig No. 1 dies in less than twenty-four hours the doses should be decreased.

3. As soon as one or more of the pigs die open the abdominal cavity aseptically.

4. Collect the peritoneal exudate into a sterile flask or tube containing about 10 c.c. of citrated salt solution.

5. Mix by shaking gently and pass the fluid through a sterile Berkefeld or Chamberland filter.

6. Again select three guinea pigs of the same weight and inject them intraperitoneally as follows:

Pig No. 1 a sublethal dose of *B. typhosus* as determined in step 2.

Pig No. 2. same dose of *B. typhosus* as for pig No. 1 and 3 c.c. of filtrate (see 5).

Pig No. 3. 6 c.c. of filtrate.

Note and explain results.

Smears should be made from the exudate of all pigs autopsied, stained with Wright's stain (see Exercise 10 B.) and carefully examined to see what has taken place.

Questions. **1.** What substances have been instrumental in bringing about the results noted?

2. Can an animal be immunized against such substances?

3. Name an important animal disease in which the substances encountered are used in producing immunity.

Method B. **1.** Immunize guinea pigs with *Staphylococcus pyogenes aureus* or *B. typhosus* (see Exercise 10).

2. Fourteen days or more after the last injection secure sera from immunized and normal pigs (see Exercises 12 and 13). Do not inactivate.

3. Place sera in sterile containers, label properly and place them in an ice sheet until needed.

4. Prepare a leukocytic emulsion by injecting one or more guinea pigs intraperitoneally with 7 c.c. of a sterile 25% solution of peptone or aleuronat solution if available. Twenty-four hours after the injection kill the pigs, open the peritoneal cavity aseptically, and collect the exudate into sterile citrated salt solution.

5. Place the suspension in sterile centrifuge tubes and centrifuge until the supernatant fluid is clear.

6. Replace the supernatant fluid with physiological salt solution and proceed to wash the cells as described in Exercise 13.

7. When washing is complete make up a suspension which has an opacity equal to that of tube No. 5 of McFarland's nephelometer.

Note. The preparation of the leukocytic extract should not begin more than forty-eight hours before it is to be used.

8. Prepare a suspension of the same organism as that used in immunizing the guinea pigs proceeding as in the prep-

aration of a bacterin. It is not necessary to kill the organisms.

9. Make up the suspension so that it compares in opacity with tube No. 2 of the nephelometer.

10. Select three capillary pipettes fitted with rubber nipples and charge them as described in Exercise 10 B. with the following ingredients:

Pipette No.	1	2	3
Leukocytic suspension..........	1 volume	1 volume	1 volume
Bacterial suspension............	1 volume	1 volume	1 volume
Immune serum..................	1 volume	none	none
Normal serum..................	none	1 volume	none
.85% salt solution..............	none	none	1 volume

11. Mix in small sterile test tubes or on concave slides as described in Exercise 10 B., draw up mixture, seal capillary end of pipette in flame being careful not to heat the suspension, and place in incubator at 37° C. for fifteen minutes.

Note. If *B. typhosus* is used, incubate for ten minutes only to avoid agglutination or bacteriolysis, since typhoid immune sera are rich in antibodies causing such reactions.

12. After proper incubation period break off the sealed tips with a file and make films from each pipette and stain. (see Exercise 10 B.).

13. Examine and compare the various preparations under the microscope. Note and explain the results.

14. What agents are instrumental in bringing about these results?

15. Is there any difference between normal and immune serum? To what is it due?

16. What effect have washed leukocytes suspended in salt solution on bacteria? Is there any explanation for this?

17. Would inactivation of the sera alter the results?

Note. If desired, rabbits may be used in place of guinea pigs.

REFERENCES

SIMON, S. E.: Infection, Immunity and Serology (1915), p. 220–227.

KOLMER, J. A.: Infection, Immunity and Specific Therapy (1917), pp. 177–189, 190–209.

McFARLAND: Pathogenic Bacteria and Protozoa, Seventh Edition, p. 307.

EXERCISE 12. DARK FIELD EXAMINATION

The dark field apparatus is made use of in the examination of organisms while viable and motile and unstained to determine their true morphology and differential characteristics.

Apparatus. Microscope; funnel for oil immersion objective; dark field condenser; slides; cover glasses; capillary pipettes; arc light or 150 watt nitrogen bulb; sterile 0.85% saline solution.

Cultures. *Treponema pallidum;* smears from teeth.

Method. **1.** The material to be examined should be placed on a thin clean slide and a clean thin cover glass placed upon it. The film of material between the slide and cover glass should be as thin as possible and free from air bubbles. It is advisable to seal the edge of the cover glass with vaselin to prevent evaporation from the mount.

2. The dark field condenser is placed below or above the stage (depending upon type of condenser used) and adjusted by means of lateral adjustment screws when using substage condenser and by hand when using condenser on top of stage.

3. Using a low power objective and looking down the tube of the microscope, move the condenser backward and forward until a brightly illuminated ring is brought to the

center of the field. The field should appear uniformly illuminated when seen through the oil immersion objective if the light is placed at the proper focal distance.

4. A drop of immersion oil is placed on the bottom of the slide just underneath the cover glass and a second drop on the cover glass.

5. Place slide on the condenser gently so as to prevent the formation of air bubbles.

6. Using the oil immersion objective, lower the tube until the lens touches the oil, then gently raise and lower the tube until the field is illuminated with bright spots. The illumination of the objects against the background depends upon the deviation of the light rays reflected by the concave mirror. The rays pass obliquely through the film of liquid which is placed between the slide and cover glass and cannot enter the objective. The organisms held in suspension in the preparation are illuminated from the side while the background is obscure.

Note. Clean slides and cover glasses are absolutely necessary in this work as foreign particles are very misleading.

7. Give results and conclusions in full.

REFERENCES

Besson, A.: Practical Bacteriology, Microbiology and Serum Therapy (1913), pp. 123–129.

McFarland: Pathogenic Bacteria and Protozoa, Seventh Edition, p. 146.

Marshall: Microbiology, Second Edition, pp. 717–721.

Nuttall: Blood Immunity and Relationship (1904).

Zinsser: Infection and Resistance (1917), pp. 391, 396, 341–384.

EXERCISE 13. THE PRODUCTION OF A HEMOLYTIC SERUM

For this work a rabbit will be immunized to washed sheep blood cells.

Apparatus. Sterile physiological salt solution; glass beads; small sterile glass funnel with cotton filter; two 200 c.c. Erlenmeyer flasks; five or six sterile centrifuge tubes; sterile 5 c.c. pipette with rubber bulb, or capillary pipette attached to rubber hose of a suction pump for draining off serum and salt solution in centrifuge tubes; sterile 14 gage $2\frac{1}{2}$ inch hypodermic needle; 5 c.c. syringe with 22 or 23 gage hypodermic needle; small curved scissors; 50° alcohol; sheep; rabbit.

Note and steps 2, 3, and 4 no change.

5. Defibrinate by agitating three or four minutes and filter through cotton.

6. Place blood in sterile centrifuge tubes and centrifuge.

7. Draw off clear serum by means of 5 c.c. pipette or suction pump.

8. Restore volume with salt solution and mix with cells by pouring from one tube to another several times.

9. Centrifuge again and repeat process until the cells have been washed with salt solution at least four times.

10. When washing is complete draw off salt solution, which should be absolutely clear, down to blood cells, transfer these to a sterile test tube and put in an ice chest if not used at once.

11. To produce hemolytic serum proceed as follows using the rabbit:

1st day, inject intravenously 0.75 c.c. pure sheep cells, as demonstrated.
3rd day, inject intravenously 1.5 c.c. pure sheep cells.
5th day, inject intravenously 3.0 c.c. pure sheep cells.
12th day, bleed rabbit from the heart as demonstrated by instructor, allow the blood to clot, pipette off serum

and inactivate at a temperature of 56° C. for thirty minutes. (For method of titration see Exercise No. 14, page 331).

When titrated and found satisfactory put in sterile container, add phenol as preservative to make 0.5% solution and label giving titre and date prepared.

12. The slow method of preparing hemolysin may be used in place of the one just described. This method differs from the former one in that intraperitoneal injections of increasing doses of washed blood cells are given at about four day intervals until the animal has received about five injections, then waiting about seven days and proceed as above. Aseptic precautions must be observed in both methods.

13. Name the advantages and disadvantages of each method. Why is it desirable to get the sheep cells washed free from blood serum?

14. Why is the rabbit serum inactivated? Why at 56° C.? Could any erythrocytes other than those of the sheep be used?

REFERENCES

See Exercise 14.

EXERCISE 14. TO DEMONSTRATE THE COMPLEMENT FIXATION TEST

The complement fixation test is one of the most complicated biological reactions used as a means of diagnosis in infectious diseases.

Apparatus. Guinea pig; rabbit; sheep; suspected serum (from aborting cow or other animal to be tested); small test tubes; test-tube rack; flasks; physiological salt solution; centrifuge tubes; disinfectant; syringe and needles.

Culture. *Bact. abortus* (or other organism depending on disease for which test is made).

I. TITRATION OF REAGENTS

Method. Four reagents other than the serum to be tested are required: 1, *complement;* 2, *hemolysin;* 3, *red blood cells from a sheep;* 4, *antigen.* Above components 1, 2 and 4 must be titrated before using in order to determine the amounts to be used in the tests.

1. *Complement.* This is contained in and obtained from fresh serum from a guinea pig. The complement is titrated for the purpose of determining the least amount which in the presence of a sufficient amount of hemolysin will produce complete hemolysis of a definite quantity of washed red blood cells from the sheep. This amount is spoken of as the *titre.*

2. *Hemolysin* (see Exercise 13). The source of hemolysin is inactivated serum from a rabbit that has been previously immunized to washed red blood cells from a sheep.

The selection of a rabbit and sheep is merely a matter

TITRATION OF COMPLEMENT

Tubes.		1	2	3	4	5	6	7
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution	(*A*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Hemolysin	(*B*)	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Suspension of blood corpuscles	(*C*)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement	(*D*)	0.02	0.04	0.06	0.08	0.1	0.0	0.1
Result after one-half hour	(*E*)	−	*	+	+	+	−	−

Incubate all tubes in water bath at 37° C. for one-half hour and read.

A. 0.9% salt solution.

B. 1% dilution of inactivated immune rabbit serum in salt solution.

C. 1% suspension of washed sheep-blood cells in salt solution.

D. 20% solution of fresh guinea pig serum in salt solution (0.4 c.c. complement made up to 2 c.c. with salt solution).

E. * A variation of reaction according to strength of complement,
+ = complete hemolysis.
− = no hemolysis.

of convenience. Any two animals of a different genus may be used. In the test for syphilis in man, human blood cells are usually used because more convenient to obtain in a number of laboratories.

TITRATION OF HEMOLYSIN

Tubes.		1	2	3	4	5	6	7	8
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution...	(*A*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Hemolysin.....	(*B*)	0.01	0.02	0.04	0.06	0.1	0.15	0.15	0.0
Suspension blood cells.........	(*C*)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement....	(*D*)	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1
Result after one-half hour.....	(*E*)	*	*	*	+	+	+	−	−

Incubate all tubes in water bath at 37° C. for one-half hour and read.

A. 0.9% salt solution.

B. 1% dilution of inactivated immune rabbit serum in salt solution.

C. 1% suspension of washed sheep-blood cells.

D. Titrated guinea pig serum diluted so that 0.1 c.c. contains 1.5 times the titre.

E. + indicates complete hemolysis.

− indicates no hemolysis.

* indicates a variation in the reaction according to the strength of the hemolysin.

The smallest quantity causing complete hemolysis is called the *titre.*

The inactivation is accomplished by heating to a temperature of 56° C. for one-half hour to destroy the complement. If not used for several days it is not necessary to heat, as complement is destroyed on standing. If it is to be kept for some time, preserve by adding 5% phenol sufficient to make a 0.5% solution. It is then titrated to determine the smallest quantity which will bring about a complete solution of the same quantity of washed sheep blood cells used in the titration of the complement, when in the presence of a proper quantity of complement.

3. *Antigen.* Antigen is an extract of the specific bacteria

made by growing the bacteria on agar and washing off with a few cubic centimeters of salt solution, and is preserved with phenol sufficient to make 0.5% and glycerin sufficient to make 1%. The suspension is placed in a shaking machine for three hours a day for three consecutive days to obtain homogeneity.

A titration of this reagent is made to determine the smallest quantity that will prevent hemolysis in the presence of 1.5 times the titre of complement and the hemolysin, sheep cells and immune serum. In other words, we must determine the smallest quantity of antigen that will fix the amount of complement used in the test.

TIRATION OF THE ANTIGEN

Tubes.		1	2	3	4	5	6	7	8	9	10	11
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution.....	(*A*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Positive serum...	(*B*)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00
Antigen.........	(*C*)	0.01	0.02	0.05	0.1	0.15	0.2	0.25	0.15	0.2	0.25	0.3
Complement.....	(*D*)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Incubate for half an hour in a water bath at 37° C., then add the hemolytic system as follows:

HEMOLYTIC SYSTEM

Tubes.		1	2	3	4	5	6	7	8	9	10	11
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Hemolysin........	(*E*)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Suspension of blood cells...........	(*F*)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Results after incubation.......	(*G*)	*	*	−	−	−	−	−	+	+	+	+

Incubate half an hour in a water bath at 37° C. and then keep in the ice box for twelve hours and read.

A. 0.9% salt solution.
B. Inactivated serum known to contain antibodies.
C. Suspension of a culture of suspected bacteria, carbolized.
D. Titrated guinea pig serum diluted so that 0.1 c.c. contains 1.5 times the titre.
E. Immune rabbit serum of known titre diluted so that 0.1 c.c. contains three times the titre.
F. 1% suspension of washed sheep blood cells in salt solution.
G. * signifies a variable reaction according to the activity of the antigen.
+ signifies a complete hemolysis.
− signifies no hemolysis.

The smallest quantity of antigen (in combination with antibody) that completely fixes the complement is known as the *titre*.

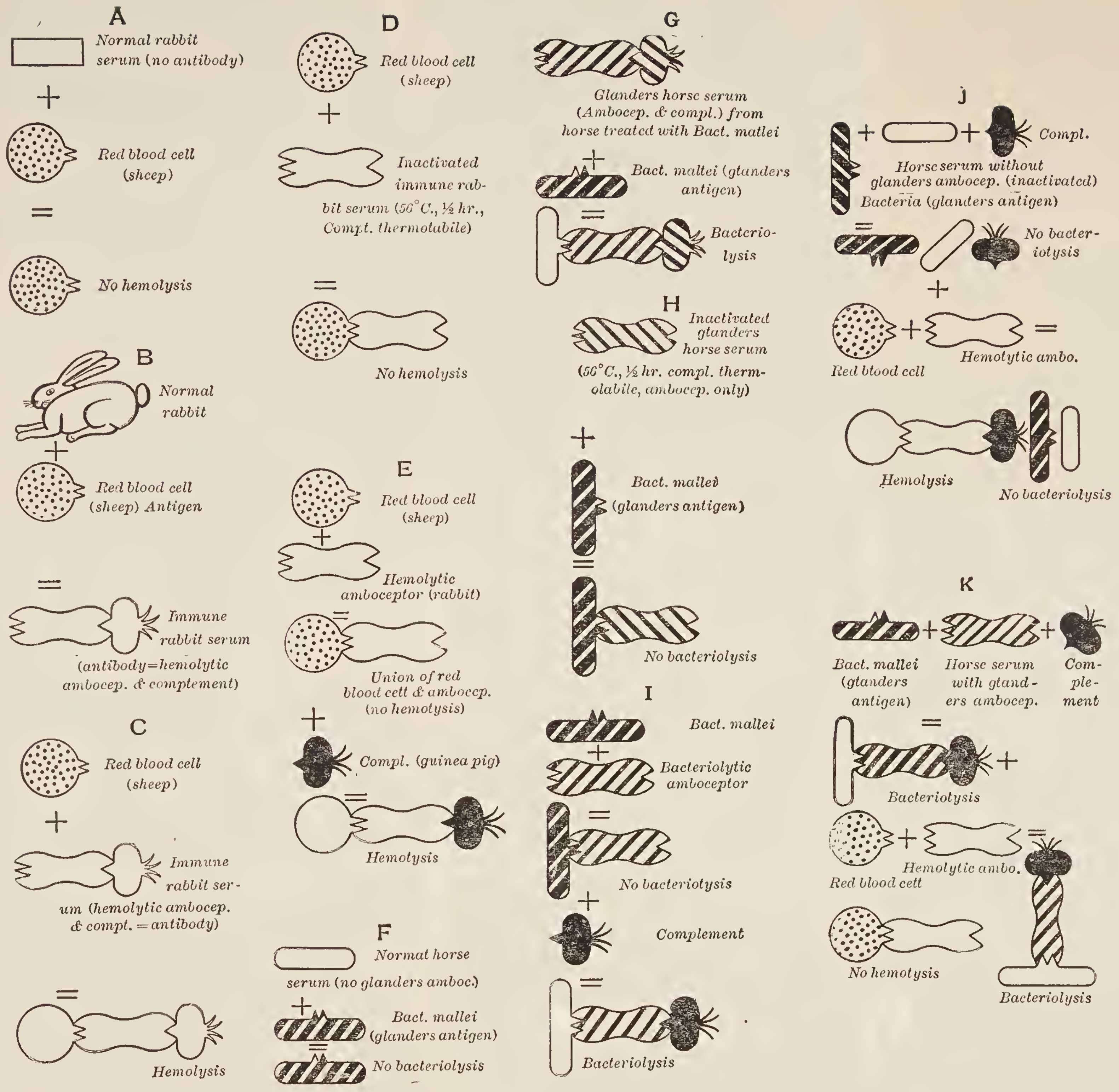


Fig. 73.—Diagrammatic Representation of Ehrlich's Side Chain Theory Applied to Cytolysis and the Complement Fixation Test. (Orig. Giltner.)

(To face page 332.)

II. COMPLEMENT FIXATION TEST. (Test proper)

1. *Suspected Serum.* This is drawn from the animal that is suspected of being infected with the infectious disease in question.

The blood is drawn from the jugular vein. It is allowed to clot and the serum collected. It must be inactivated before testing unless it is to be held for a week or more before applying the test, in which case inactivation is not necessary, but 1% phenol should be added as a preservative.

Note. The test proper and controls must be run *at the same time. If several tests are run at the same time one set of controls is sufficient.*

2. *Test of Suspect Serum.*

Tubes.		Test Proper.				Controls.				
		1	2	3	4	1 *	2 *	3 *	4 *	5
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Salt solution......	(*A*)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Suspect serum.....	(*B*)	0.1	0.02	0.04	0.04	0.0	0.0	0.0	0.0	0.5
Antigen..........	(*C*)	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0
Complement......	(*D*)	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.1

* In routine work it is not necessary to run these controls again.

3. Then add the hemolytic system.

Tubes.		Test Proper.				Controls.				
		1	2	3	4	1 *	2 *	3 *	4 *	5
		c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Blood cells.......	(*E*)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Hemolysin.......	(*F*)	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1

Incubate one-half hour at 37° C. in water bath and then place in ice box for twelve hours and read.

A. Salt solution, 0.9%.
B. Suspect serum inactivated for one-half hour.
C. Antigen, two times titre.
D. Complement, 1.5 times titre.
E. 1% washed sheep blood corpuscles in salt solution.
F. Immune rabbit serum (hemolysin) diluted so that 0.1 c.c. contains three times the titre.

* In routine work it is not necessary to run these controls again.

Control tubes 2 and 3 should show complete hemolysis. Control tubes 1 and 4 should show complete absence of hemolysis. Control tube 4 is control on the inactivation of the suspect's serum and should show absence of hemolysis.

Hemolysis in the other tubes indicates the absence of antibodies in sufficient quantity in the amount of serum used to fix complement.

4. Give all results in detail and draw conclusions.

REFERENCES

Mohler, J. R. and Eichhorn, A.: The diagnosis of glanders by complement fixation test. Bul. 136, B. A. I., U. S. Dept. Agr. (1911).

Surface, F. M.: The diagnosis of infectious abortion in cattle. Bul. 166, Kentucky Agr. Expt. Sta. (1912).

Hadley, F. B. and Beach, B. A.: The diagnosis of contagious abortion in cattle by means of the complement fixation test. Res. Bul. 24. Wis. Agr. Expt. Sta. (1912).

Mohler, John R., Eichhorn, A. and Buck, J. M.: The diagnosis of dourine by complement fixation. Jour. Agr. Res. Bul., Vol. I (1913), pp. 99–109.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 354–533, 455, 476, 511–515.

Zinsser: Infection and Resistance (1914), pp. 134–217.

EXERCISE 15. TO DEMONSTRATE THE PRECIPITIN TEST

This test is based upon the fact that, if an animal is immunized with any foreign protein, its serum acquires the property of precipitating that particular protein even when in very high dilutions.

Its most important applications are:

1. To identify blood stains and to detect adulteration of meat products in medico-legal cases. To differentiate between types of bacteria in routine and research work.

2. In order to perform this test on one or more unknown proteins, it is necessary that a specific immune

serum be produced, by animal inoculation for each protein suspected.

Example. If a sample of blood is to be identified and there are reasons for believing that it orginated from a man, dog or horse, it will be necessary to immunize one rabbit to human, one to canine, and one to equine blood or serum.

The immunizing agent must be sterile, and it is well to immunize more than one rabbit to each individual sample, because many times the animal will die from an anaphylactic shock after a few injections have been given. One should, therefore, draw a small sample of blood after the third injection and test it for potency. If it shows a sufficiently high titre, no further injections should be attempted.

Apparatus. 10 c.c. syringe with 22 or 25 gauge needle; one 19 gauge aspirating needle; 100 c.c. wide mouth glass container; two rabbits; sterile horse and cow serum; fourteen flasks 100 c.c.; fourteen small test tubes; six 1 c.c. and two 5 c.c. pipettes; disinfectant; sterile physiological salt solution; ether.

Method. Inject as many rabbits as required according to the following schedule:

1st day 2.5 c.c. sterile cow serum intravenously,
3rd day 5 c.c. sterile cow serum intravenously,
5th day 10 c.c. sterile cow serum intravenously.

On the 12th day after the last infection, bleed the rabbit from the heart as follows:

1. Put the animal in the dorsal position on a suitable animal board, securing it in such manner that the operation may be performed without any interference. Sufficient ether may be administered to keep the animal quiet, without putting it entirely under anæsthesia.

2. Clip the hair from an area about 3 cm. in diameter over the region of the left side of the thoracic cavity, beginning about 0.5 cm. to the left of the median line of the sternum, and disinfect with 5% phenol.

3. With the tip of the left middle finger, locate the place

of maximum pulsation and, placing the point of the needle on the spot where the beat of the heart is most plainly felt, insert the needle just through the thoracic wall. Do not stab blindly at the heart, but holding the needle loosely between the tips of the thumb and forefinger of the right hand, move it about carefully until the heart can be felt beating firmly against the point of the needle.* Now thrust the needle into the heart and, if no blood comes, withdraw the needle slightly until the blood flows freely. If venous blood appears, the right ventricle or auricle has been entered. It is better that the needle enter the left ventricle near the apex. Familiarity with the anatomy of the thoracic cavity and its organs is of great importance in performing this operation successfully.

4. Collect the blood in a wide mouth glass container, and place it in a slanting position at room temperature until the blood has clotted and the serum begins to separate from the clot. Now place the container in the ice chest in an upright position and draw off serum after eighteen to twenty-four hours.†

5. Place the clear serum in several small, sterile glass bulbs holding 1 to 2 c.c., and seal the bulbs in a small flame, using care to avoid heating the serum. Serum collected in this way and placed in a cool, dark place will retain its precipitating properties for several months.‡

* Needles used for this purpose should have a short bevel, preferably 2 mm. in length.

† As it is necessary to have a clear serum for this work, the rabbit should be bled after a period of fasting. If bled shortly after being fed, the serum will possess an undesirable opalescence.

‡ In place of using the intravenous method for the production of precipitin one may inject intraperitoneally slightly larger doses at five day intervals until about six injections have been given. Defibrinated blood may also be given in place of clear serum; this, however, results in the production of hemolysins as well and such substances are not desirable, as they would interfere with the complement fixation reaction in case one should wish to apply that test for additional information.

6. In five sterile 100 c.c. flasks make dilutions of cow serum as follows:

Flask I.	0.1 c.c. serum +9.9 c.c. +0.85% NaCl sol.		=1 : 100	Dilution
Flask II.	1.0 c.c. from flask I	+4.0 c.c. 0.85% NaCl sol.	=1 : 500	Dilution
Flask III.	1.0 c.c. from flask I	+9.0 c.c. 0.85% NaCl sol.	=1 : 1000	Dilution
Flask IV.	1.0 c.c. from flask II	+9.0 c.c. 0.85% NaCl sol.	=1 : 5000	Dilution
Flask V.	1.0 c.c. from flask III	+9.0 c.c. 0.85% NaCl sol.	=1 : 10,000	Dilution

7. Set up seven tubes and add reagents according to the following table:

THE PRECIPITIN TEST

Tube No.	1	2	3	4	5	6	7
Cow serum diluted..	2 c.c. 1–100	2 c.c. 1–1500	2 c.c. 1–1000	2 c.c. 1–5000	2 c.c. 1–10000	0	2 c.c. 1–1000
0.85% NaCl solution..	0	0	0	0	0	2 c.c.	0.1 c.c.
Precipitin...........	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1	0
Reaction............	+	+	+	±	±	–	–

+ Signifies precipitate. No precipitating serum should be used in an actual test if it does not show reaction in a dilution of 1 : 1000.

± Reaction depends upon potency of precipitin.

– Controls, no precipitation.

8. The precipitin must be added very slowly and allowed to run down the side of the tube and collect on the bottom. Do not shake the tubes. Precipitate will form almost immediately. Observe for four to five minutes and then place in incubator for twenty minutes and note results. Any cloudiness appearing after this period should be disregarded.

9. Set up another series of tubes, using horse serum in place of cow serum, and note any difference in the results obtained.

10. Give results and conclusions in full.

REFERENCES

McFarland: Pathogenic Bacteria and Protozoa, Seventh Edition, p. 146.

Marshall: Microbiology, Second Edition, pp. 717–721.

Nuttall: Blood Immunity and Relationship (1904).

Kolmer: Infection, Immunity and Specific Therapy (1915), pp. 70, 71, 314–337, 892, 920.

Zinsser: Infection and Resistance (1917), pp. 391, 396, 341, 384.

EXERCISE 16. DETERMINATION OF THE VIRULENCE OF BACTERIUM DIPHTHERIÆ

Apparatus. Eight tubes Loeffler's blood serum; 2 c.c. broth; two tubes glucose agar; or two tubes glucose blood agar; 3 c.c. sterile physiological salt solution; two guinea pigs; 1000 units diphtheria antitoxin; 1 c.c. diphtheria toxin; Loeffler's methylene blue; small sharp hypodermic needle.

Method. **1.** Swab the throat and nose of a patient suffering with diphtheria, using a sterile swab for each. If a diphtheria patient is not available, each student should swab his own throat and nose then dip the swab into a light suspension of diphtheria organisms.

2. Smear the swabs on tubes of Loeffler's blood serum. Incubate at 37° C. for twelve to twenty-four hours.

3. Make a microscopic examination for *Bact. diphtheriæ* as follows: With a platinum loop transfer a small portion of the typical growth to a glass slide and air dry. Stain with Loeffler's methylene blue for one and one-half minutes; wash, dry and examine.

4. If typical organisms are present, add 1 c.c. of sterile broth to each tube and emulsify the growth with a platinum loop.

5. Take one loopful of this emulsion and streak over the surface of a glucose agar or glucose blood agar plate. Incubate twenty-four hours at 37° C.

6. After twenty-four hours incubation plates should show characteristic colonies. Transfer one or more characteristic colonies to Loeffler's blood serum or other suitable medium and incubate for twenty-four hours at 37° C.

7. At the end of this time emulsify the resultant growth in 3 c.c. of sterile physiological salt solution.

8. Shave the hair from the abdomen of two guinea pigs weighing about 250 grams each.

9. At the prepared site inject intracutaneously 0.15 c.c. of

this suspension into each of the two guinea pigs. (As many as six cultures may be tested in this way on two animals.)

10. Inject intraperitoneally or intracardially one of the guinea pigs at the same time with about 500 units of diphtheria antitoxin.

11. Another method that may be used is to inject 2 c.c. of a 48 hour dextrose broth culture subcutaneously into a normal guinea pig. As a control inject the same amount of culture into a guinea pig which has been previously treated with 500 units of antitoxin.

Schick Reaction

12. Dilute a standard toxin with a 0.5% phenol solution so that 0.1 c.c. contains $\frac{1}{50}$ of a M. L. D.

13. Inject intradermally into the right forearm 0.1 c.c. of the solution prepared in step 12.

14. As a check inject 0.1 c.c. of sterile physiological salt solution intradermally into the left forearm.

15. Define the diphtheria toxin and antitoxin unit. Is it the same as the unit for tetanus toxin? Define each and point out the difference. What do M. L. D; Lo; and L+ denote?

16. If diphtheria organisms are present in the patient, how is it indicated in the guinea pig inoculation?

What is the character of a positive reaction and the significance of the Schick test?

REFERENCES

Marshall: Microbiology, Second Edition, p. 379.

Kolmer: Infection, Immunity and Specific Therapy (1917), pp. 228–232, 894.

Zinsser: Infection and Resistance (1917), pp. 41, 107, 131–133.

EXERCISE 17. ISOLATION, AND IDENTIFICATION OF TYPHOID DYSENTERY GROUP

Apparatus. Six agar slants; six Dunham's peptone solution; six litmus milk; six lead acetate triple sugar agar slants; eleven triple sugar agar slants; six saccharose, six dextrose, six lactose, six maltose, and six mannite broth fermentation tubes; nine Endo plates; agglutination tubes, L-shaped glass rod; nine Petri dishes.

Cultures. *B. typhosus*, *B. paratyphosus*, "A," *B. paratyphosus*, "B," *Bact. dysenteriæ*, "Y," *Bact. dysenteriæ*, Flexner, *Bact. dysenterinæ*, Shiga.

A. Studies of Known Organisms.

Method. 1. Inoculate the following media with the above organisms in order to observe the reaction on each. Endo's medium (use glass rod bent at a right angle, see Exercise 1, step 2 under Water Analysis) agar slants. Dunham's peptone solution, litmus milk, triple sugar slants, lead acetate triple sugar slants, and fermentation tubes of saccharose, lactose, dextrose, maltose and mannite broth.

2. Incubate at 37° C. and record results at the end of twenty-four and forty-eight hours.

3. Make up a suspension of each organism at the end of twenty-four hours from the triple sugar agar slants. (The density of a standard suspension is such that the outlines of a glass rod held behind the tube can just be seen through the suspension.)

4. Set up agglutination tests as follows:

Tube.	Sterile NaCl	Serum 1 : 100	Suspension of organism.	Dilution.
1	1.0 c.c.	0	2 to 4 drops	Control
2	1.8 c.c.	0.2 c.c.	"	1 : 1000
3	1.0 c.c.	1.0 c.c. from No. 2	"	1 : 2000
4	1.0 c.c.	1.0 c.c. from No. 3	"	1 : 4000
5*	1.0 c.c.	1.0 c.c. from No. 4	"	1 : 8000

* Discard 1 c.c. from tube No. 5 after it has been mixed.

In agglutinating for dysentery use following:

Tube.	Sterile NaCl	Serum 1 : 100	Suspension of organism.	Dilution.
1	1.0 c.c.		2 to 4 drops	Control
2	——	2.0 c.c.	"	1 : 100
3	1.0 c.c.	1.0 c.c. from No. 2	"	1 : 200
4	1.0 c.c.	1.0 c.c. from No. 3	"	1 : 400
5	1.0 c.c.	1.0 c.c. from No. 4	"	1 : 800
6*	1.0 c.c.	1.0 c.c. from No. 5	"	1 : 1600

* Discard 1 c.c. from tube No. 6.

B. Study of Unknown Organisms from Feces, Urine and Blood.

5. Inoculate an Endo plate from a sample of feces, blood and urine suspected to contain some of the typhoid-dysentery organisms. If no suspected specimens are available, normal feces, blood and urine may be inoculated with any of the above cultures and handed to the student as an unknown.

Note. Always inoculate triple sugar agar slants direct from urine and blood at the same time Endo plates are inoculated, as pure cultures are often obtained. This will reduce the time twenty-four hours.

6. Incubate all cultures twenty-four hours at 37° C.

7. Fish at least three small transparent colonies (bluish when viewed by transmitted light against a dark background) from Endo's medium and transfer to triple sugar.

8. Incubate triple sugar agar slants twenty-four hours at 37° C. If the reaction is typical, prepare a suspension as in step 3 and run agglutination tests as in step 4.

9. Draw a chart showing the action of each organism upon the fermentable substances. May the various strains of dysentery be differentiated by this chart? Can the other organisms of the typhoid-dysentery group be identified in this way?

10. In confirming an unknown organism in this group, what should be your final test? Why was lead acetate added to the triple sugar agar slant? Is this a reliable test?

12. Why are the dilutions higher for the typhoid group than for the dysentery group? What is cross agglutination?

REFERENCES

Hiss and Zinsser: A Textbook of Bacteriology, Chapters XV and XVI.
Stitt: Practical Bacteriology, Blood Work and Animal Parasitology, pp. 186–190.

TECHNIC FOR STANDARDIZATION OF BACTERINS AND BACTERIAL VACCINES

A. Wright's Method.

Apparatus. Capillary pipette with rubber nipple; three concave slides; three or more properly cleaned slides; sharp needle or pin; cotton; 50% alcohol; citrated salt solution; ocular to which a diaphragm has been fitted; Wright's stain.

1. Make a diaphragm by cutting out a circular piece of stiff paper or cardboard (thin) to fit into the upper part of the ocular of the microscope. In the center of this piece cut a small hole not more than 1 mm. square.

2. Pour about 1 c.c. of bacterial suspension and citrated salt solution on concave slides labelled correspondingly.

3. Put a wax pencil mark about two centimeters from the point of the capillary pipette.

4. Disinfect the skin of the left thumb just above the root of the nail with 50% alcohol, let dry, tie a handkerchief tightly around the thumb in the region of first phalanx and flex the thumb.

5. Heat the point of the needle in a flame, allow to cool and puncture the skin of the left thumb 2 mm. above the root of the nail.

6. Charge the capillary pipette in the following order with one to three volumes of citrated salt solution, one volume of blood and one volume of bacterial suspension.

Note. The heavier the suspension is the more citrated salt solution should be used in order to facilitate the counting. In case the bacterial

suspension is very thin, one may replace the citrated salt solution with two or three volumes of bacterial suspension, making due allowances when estimating the number of bacteria per c.c. of suspension by dividing by two or three according to the number of volumes taken.

7. Mix the contents of the pipette on a clean concave slide by expelling and drawing up repeatedly. To bring about thorough mixing, the mixture should be drawn up in a solid column and no air bubbles allowed to form.

8. Place small drops of this suspension near one end of several clean plain slides, and make smears as follows: lay the slide on a flat surface, the drop at the right end, steady it with the forefinger and thumb of the left hand, take another slide between thumb and middle finger of right hand, placing it at an angle of 30 degrees, the end resting upon the first slide touching the left side of the drop. With a quick steady motion push the slide in right hand toward the fingers of the left. The film must be made in one motion to obtain a single uniform layer. Let the film dry in air and do not fix in the flame.

9. Flood the slide with a known quantity of Wright's stain and let stand one minute, then add an equal volume of water and let stand two more minutes. Rinse off the stain gently and allow to dry.

10. Place the slide under the microscope and through the diaphragm in the ocular count the red cells and bacteria in several fields until five hundred or one thousand of the former have been counted.

Note. If the bacteria or blood cells are clumped, the film should be discarded as no reliable counts can be made from such preparations. One c.c. of normal blood contains about five billion red cells. In the suspension bacteria and red cells are present in known proportions, therefore:

$$\text{Red cells counted} : \text{bacteria counted} :: \text{five billion} : x.$$

From this equation, the number of bacteria per c.c. of bacterial suspension can be estimated.

Example:

Red cells counted... 1000
Bacteria counted... 1500
1000 : 1500........ 5,000,000,000 : x

$$x=\frac{5{,}000{,}000{,}000\times 1500}{1{,}000}$$ or 7,500,000,000 bacteria per c.c. suspension.

From a known suspension dilutions can be made to obtain the desired number of bacteria per c.c. of suspension.

B. Hopkin's Method.

Note. This method is based upon the concentration of a bacterial suspension or culture by centrifugation and the preparation of standard emulsions from the sediment.

Apparatus. Sterile Hopkin's vaccine tube; sterile rubber caps; sterile physiological salt solution; containing 0.5% phenol; small funnel with cotton filter; sterile capillary pipette with rubber nipple.

Method. **1.** Filter washing from slant cultures through cotton filter into a Hopkin's vaccine tube, cover with a rubber cap and centrifuge for one hour at about 2800 revolutions per minute.

2. Remove the salt solution and sediment down to the 0.05 mark and add 5 c.c. of physiological salt solution. This will make a one per cent suspension.

3. Force the bacteria into suspension by means of a sterile capillary pipette fitted with a rubber nipple.

4. Transfer the suspension to a sterile test tube and kill. See par. 8, Ex. 10.

Note. The following shows the results of carefully counted suspensions obtained in this way:

	Per Cent.	Billion per c.c.
Staphylococcus aureus and albus	1	10.0
Streptococcus hemolyticus	1	8.0
Gonococcus	1	8.0
Pneumococcus	1	2.5
Bacillus typhosus	1	.8.0
Bacillus coli	1	4.0

In applying this method in the standardization of bacterins, it should always be borne in mind that the speed of the centrifuge determines the standardization of the suspension. It is, therefore, necessary to have a good centrifuge for this work.

REFERENCES

Simon, S. E.: Infection Immunity and Serology (1915), pp. 196–198.
Kolmer, J. A.: Infection, Immunity and Specific Therapy (1917), pp. 210–223.

APPENDIX

OUTLINE FOR THE STUDY OF MICROBIOLOGY *

I. MORPHOLOGICAL AND CULTURAL MICROBIOLOGY

A. Morphology and Development.
  1. Gross anatomy.
    a. Form.
    b. Size.
    c. Arrangement or grouping.
    d. Multiplication.
    e. Involution, variability and mutation.
  2. Histology of cell.
    a. Wall or outer membrane.
    b. Capsule.
    c. Protoplasm, beaded forms, granules.
    d. Nuclear material.
    e. Flagella and motion.
    f. Spores.
  3. Classifications and their basic features.

B. Cultural Significance.
  1. Media.
    a. For morphologic and developmental studies.
    b. For cultural effects.
  2. Colonies.
  3. Cultural features.
  4. Biochemical features.

*Adapted from Marshall, *vide* 43d Annual Report of Michigan State Board of Agriculture.

C. Staining Values.
   1. Demonstrations of parts of cell.
   2. Identification of species.
   3. Differentiation of species.

D. Determination of Microorganisms.
   1. Methods employed.
   2. Differential characteristics.

II. PHYSIOLOGIC MICROBIOLOGY

A. Cell Studies.
   1. Composition of cell contents.
   2. Composition of cell wall.
   3. Physical products of physiological significance.
      a. Heat.
      b. Light.
   4. Products of physiological significance of which little is known.
      a. Pigment.
      b. Enzymes.
      c. Aromatic compounds.
      d. Toxins.
   5. Absorption or assimilation of foreign bodies.
   6. Chemotaxis.
   5. Phototaxis.
   8. Aerotaxis.
   9. Plasmolysis.
   10. Plasmoptysis.

B. Studies in Metabolism.
   1. Elements required in growth of microorganisms.
   2. Respiration.
   3. Nutrition.
   4. Moisture.
   5. Temperature of cultivation.
   6. Conditions of media; reaction, composition, etc.

7. Physiologic test media.
8. Identification and determination of species of micro-organisms by means of
    - *a.* Cultural physiologic methods.
    - *b.* Chemical tests.
    - *c.* Physical tests.
    - *d.* Biological tests.
9. Enzymes.

C. Studies in Association.

1. Symbiosis.
2. Metabiosis.
3. Antibiosis.

D. Common Fermentative Changes Produced by Micro-organisms.

1. Studies in enzymes.
    - *a.* Formation.
        - Zymogen.
        - Activator.
        - Kinases.
    - *b.* Kinds.
    - *c.* Actions (*specificity*) and materials fermented.
    - *d.* Conditions under which enzymes act;
        - (1) Physical.
            - Temperature.
            - Radiation, light rays (solar, electric, etc.), Röntgen rays, radium rays and emanations.
        - (2) Chemical and physico-chemical.
            - Activators; kinases, organic acids, bases, neutral salts.
            - Protective agents.
            - Paralysors and poisons.
            - Concentration of solutions.
            - Reaction of substrate.
            - Extent of accumulated products.

2. Products manufactured by fermentation.
    a. Necessary and limiting conditions of production.
    b. Most favorable conditions of production.
    c. Methods of determination.
        Qualitative.
        Quantitative.
    d. Constancy and variability of products.
    e. Gradation in fermentation changes.
        Intermediate products.
        Ultimate products.

E. Products Significant through the Intermediation of a Host.

1. Antigens.*
    a. Cells.
    b. Cell products.
        Toxins, diffusible and endotoxin.
        Bacterial proteins.
        Enzymes.
2. Antibodies.*
    a. Antitoxin.
    b. Agglutinins.
    c. Precipitins.
    d. Opsonins.
    e. Aggressins.
    f. Cytolysins.
    g. Anaphylactins.

F. Influencing Agents and Their Effects.

1. Light.
    a. Direct.
    b. Diffuse.
    c. Special.

* See pp. 65–67, 150, 161, Kolmer's Infection, Immunity and Specific Therapy (1917).

- *d.* Phototropism.
- *e.* Phototaxis.

2. Temperatures.
   - *a.* Heat.
     - Direct flame.
     - Dry.
     - Moist.
     - Steam under pressure.
   - *b.* Cold.
   - *c.* Thermotaxis.
   - *d.* Thermotropism.
3. Electricity.
4. Desiccation.
5. Mechanical pressure.
6. Mechanical agitation.
7. Gravity.
8. Chemicals.
   - *a.* Chemotropism.
   - *b.* Chemotaxis.
   - *c.* Concentrated solutions.
   - *d.* Antiseptics, disinfectants.

III. HYGIENIC MICROBIOLOGY

A. Communicable Diseases of

1. Man and animals.
   - *a.* Causal agent or microorganism.
   - *b.* History of microorganism.
   - *c.* Vitality or persistency of microorganism.
   - *d.* Means of dissemination and avenues of infection.
   - *e.* Distribution of microorganism in body.
   - *f.* Management of disease.
   - *g.* Prevention of disease.
   - *h.* Care of dead from communicable diseases.

B. Surgical Significance.

1. Wounds.
2. Abscesses.
3. Septicemia and pyemia.
4. Malignant growths.
5. Operations.

C. Susceptibility and Immunity.

1. Natural.
    a. Race.
    b. Species.
    c. Age.
    d. Individual idiosyncrasies.
    e. Body components.
2. Acquired, active or passive.
    a. Devitalization.
    b. Hereditary predisposition.
    c. One attack of disease.
    d. Vaccines.
    e. Bacterins.
    f. Toxins.
    g. Other bacterial products.

D. Serum Therapy—Microbial Therapeutics.

1. Diagnostic agents.
    a. Tuberculin.
    b. Mallein.
    c. Bacterial suspensions.
    d. Diphtheria toxin (Schick).
    e. Luetin.
2. Remedial agents.
    a. Antitoxins.
    b. Serums.
    c. Vaccines.
    d. Bacterins.

E. Disinfection and Antisepsis.

1. Agents employed.
    a. Mode of action.
2. Determination of values, phenol coefficient.
3. Methods.

F. Sanitary Studies.

1. Water analysis.
    a. Methods.
    b. Interpretation of results.
2. Water contamination and filtration.
3. Sewage analysis.
    a. Methods.
    b. Interpretation of results.
4. Sewage destruction.
    a. Aerobic—filtration.
    b. Anaerobic—septic tank.
    c. End products.
5. Ventilation.
    a. Currents as means of dissemination.
    b. Filtration and washing of air.
    c. Germ content of air.
    d. Methods of analysis.
    e. Interpretation of results of analysis.
6. Foods.
    a. Poisonous.
    b. Infected.

IV. DAIRY

A. Milk Supply.

1. Communicable diseases conveyed through milk.
    a. Kinds of microorganisms.
    b. Avenues of transmission.
    c. Prevention.

2. Environment of animals and conditions of milking.
   a. Stabling.
   b. Feeding.
   c. Milker.
   d. Utensils.
3. Bacterial content of milk in udder.
   a. Non-pathogenic microorganisms.
   b. Pathogenic microorganisms and antibodies.
   c. Conditions of growth in udder.
   d. Abnormal udders.
4. Bacterial action on constituents of milk.
   a. Proteins.
   b. Butter fat.
   c. Lactose.
   d. Mineral constituents.
5. Analysis of air of stables.
   a. Before cleaning.
   b. Immediately after cleaning.
   c. Before feeding.
   d. Immediately after feeding.
   e. Analysis of out-door air.
6. Determination of value of staining.
7. Determination of value of aeration.
8. Determination of value of cooling.
   a. Simple cooling.
   b. Cooling and keeping cool.
   c. Cooling and warming, then cooling.
9. Cleansing of utensils.
   a. Methods and their values.
   b. Water analysis.
10. Milk control.

B. Pigment in Milk and Cheese.

1. Kinds.
2. Character.

3. Condition of formation.
4. Control.

C. Fermentations in Milk, Butter and Cheese.
  1. Kinds.
    a. Lactic.
    b. Butyric.
    c. Alcoholic.
    d. Gaseous.
    e. Peptic.
    f. Rennet.
    g. Ropy.
    h. Soapy.
    i. Taints.
      Bitter flavor, barn-yard, tallowy.
    j. Special.
      Kephir, koumiss, matzoon, leben, yoghurt, etc.
    k. Natural enzymes (galactase).
    l. Antibody formation (agglutinins, etc.).
  2. Microorganism involved.
    a. Its life history.
  3. Nature of fermentation.
  4. Constituents acted upon.
  5. Products.
  6. Conditions influencing it.
  7. Controlled or fostered.

D. Pasteurization and Sterilization.
  1. Determination of significance of each.
  2. Methods employed.
  3. Practical utilization.

E. Starters.
  1. Natural.
    a. Sour milk.
    b. Sour cream.
    c. Buttermilk.
    d. Others.

2. Artificial.
    - *a*. Pure cultures.
    - *b*. Mixed cultures.
3. Value determined.
4. Preparation.
5. Employment.
6. Constancy.
7. Influencing conditions.
8. Facts governing amounts to employ.

F. Butter.

1. Microorganisms present.
2. Microorganisms compared with those of milk.
3. Environmental condition for bacterial life changed.
4. Quality.
    - *a*. Influenced by pasteurization of the cream.
    - *b*. Influenced by growth of microorganisms.
    - *c*. Factors influencing stability.
    - *d*. Methods of preservation.
5. Decomposition.
    - *a*. Products.
    - *b*. Factors influencing.
    - *c*. Correlation between the presence of certain groups of organisms and specific flavors.
6. Significance of casein and buttermilk in butter.

G. Cheese.

1. Kinds of microorganisms employed in different cheeses.
2. The study of microorganisms in the ripening process.
3. Influence of microorganisms on aroma and flavor.
4. Keeping values.

H. Preservatives.

I. Disinfectants utilized.

V. SOIL

A. The Making of Soil.

1. Microorganisms in soil.
    a. Number at different depths and in different soils.
    b. Kinds at different depths and in different soils.
    c. Character of microorganisms found.
    d. Rate of growth.
2. Disintegration of inorganic material.
3. Decomposition of organic material.
    a. Celluloses.
    b. Starches and sugars.
    c. Proteins, etc.
4. Action of iron and sulphur bacteria.

B. Ammonification.

C. Nitrification—The nitroso- and nitro-processes.

1. Conditions influencing.
    a. Physical.
    b. Reaction.
    c. Temperature.
    d. Supply of oxygen.
    e. Amount of organic matter present.
    f. Moisture.

D. Denitrification.

1. Factors influencing the loss of nitrogen.

E. Nitrogen Fixation.

1. Symbiotic.
2. Nonsymbiotic (aerobic and anaerobic).

VI. PLANT

A. Nitrogen Accumulators.

1. Microorganism involved.
2. Cultural characteristics.
3. Formulation of nodules.
4. Character of nodules.
5. Conditions under which they form.
6. Determination of nitrogen accumulations.
7. Significance of nodules.

B. Microbial Diseases.

1. Kinds.
2. Microorganisms found as causal agents.
3. Cultural characteristics.
4. Resistance of microorganisms.
5. Persistency.
6. Methods of treatment.
7. Pathology.

C. Microbial Decomposition of Fruits, Vegetables and Other Plant Substances.

1. Nature.
2. Microorganism studies.
3. Conditions favoring.
4. Control.
5. Structural changes.

VII. FERMENTATION

A. Factors Controlling Fermentations.

1. Presence of microorganism.
2. Purity of culture.
3. Vigor of cell.
4. Character of fermentable material.
5. Air supply.

6. Reaction of medium.
7. Temperature.
8. Concentration of fermentation solutions.
9. Concentration of products of fermentation.

B. The Production of Enzymes by Microorganisms.

1. Formation of enzyme in cell.
2. Its secretion by the cell.
3. Determinative methods for study.
4. Environmental influences.

C. The Fermentations.

General.

1. The Enzymes.
    a. Hydrolytic of
        Carbohydrates = Carbohydrases.
            Cellulases.
            Hemicellulases.
            Glycogenases.
            Dextrinases.
            Inulinase.
            Saccharase.
            Lactase.
            Maltase.
            Trehalase.
            Raffinase.
            Amygdalase.
            Tannase.
            Pectase, etc.
        Fats = Esterases.
            Lipases of natural fats.
            Stearinases, etc.
        Proteins = Proteinases.
            Peptases.
            Tryptases.
            Ereptases, etc.

Acid amides = amidases.
  Urease.

b. Reducing-oxidizing = zymases, acting on
  Carbohydrates, to form alcohol and CO_2.
    Zymases of *d*-dextrose, *d*-levulose, etc.
  Carbohydrates to form lactic acid.
    Lactic acid-bacteria zymase.

c. Oxidizing = oxidases.
  Alcoholase.
  Lactacidase.
  Acetacidase.
  Tyrosinase.
  Laccase.

d. Reducing = Reductases.
  Catalase.
  Peroxidase.
  Methylen blue, indigo and azolitmin reductase.
  Perhydridase.
  Sulphur reductase.
  Nitrate and nitrite reductase, etc.

e. Coagulating.
  Caseinase.
  Parachymosin.
  Thrombase.
  Pectinase.

2. Materials Acted Upon.
  a. Celluloses.
  b. Starches.
  c. Sugars.
  d. Fats.
  e. Proteins.
  f. Organic acids, etc.
  g. Alcohols.

3. Products resulting.

Special.

1. Alcoholic.
   a. Beer and distilled liquors.
   b. Wine, cider and other fermented fruit juices.
   c. Ginger beer.
   d. Koumiss, etc.

2. Acetic acid.
   a. Vinegar.
   b. Mashes.
   c. Foods.

3. Lactic acid.
   a. Milk.
   b. Mashes.
   c. Foods, sauer kraut, brine pickles, etc.
   d. Ensilage.

4. Butyric acid.
   a. Milk.
   b. Mashes.
   c. Foods.

5. Ammoniacal.
   a. Urea, uric and hippuric acid.
   b. Proteins and their nitrogenous fractions.

6. Proteolytic.
   a. Proteins, albumins.
   b. Proteoses, albumoses.
   c. Peptones.
   d. Peptids.
   e. Amino-acids.
   f. Amins and other ammonia derivatives.
   g. Ptomains.
   h. Leucomains.
   i. Non-nitrogenous organic acids.
   j. Alcohols.
   k. Ammonia, H_2S, and other gases.

7. Nitrification.
8. Denitrification.
9. Ammonification.

VIII. FOOD AND DRINK PRESERVATION

A. Preservation of Foods.

1. Freezing.
2. Cold storage.
3. Salting.
4. Drying, evaporating or concentrating.
5. Smoking.
6. Corning.
7. Canning.
8. Chemical preservatives or antiseptics.
9. Preserving.
10. Pressure.
11. Fermentations.

B. Preservation of Drinks.

1. Pasteurizing and sealing.
2. Cold storage.
3. Chemical preservatives.
4. Carbonating.
5. Filtration.
6. Fermentation.

CLASSIFICATION OF MIGULA (MODIFIED)

Order.	Family.	Genus.	Species.	Variety.
I. EUBACTERIA (true bacteria) Suborder. A. *Haplobacterinæ.* (lower bacteria.)	*Coccaceæ* round forms	*Streptococcus* . . . division in 1 plane, no flagella	*pyogenes* *erysipelatus*	
		Micrococcus . . . . division in 2 planes, no flagella	*tetragenus*	
			pyogenes	*aureus* *albus*
		Sarcina . . . . division in 3 planes, no flagella	*lutea*	
		Planococcus . . . . division in 2 planes, flagella	*agilis*	
		Planosarcina . . . division in 3 planes, flagella	*mobilis*	
	Bacteriaceæ rod forms.	*Bacterium* . . . . . . (straight rods) (non-flagellate)	*lactis acidi* *bulgaricum* *aerogenes* *abortus* *tuberculosis*	
		Bacillus . . . . (straight rods) (flagellate)	*fluorescens liquefaciens* *mycoides* *prodigiosus* *typhosus* *coli*	
		Pseudomonas . . . (straight or irregular rods, polar flagella)	*radicicola* *campestris*	
	Spirillaceæ curved (comma) or spiral forms.	*Spirosoma* . . . . . comma to spiral forms, stiff, no flagella.	*nasale*	
		Microspira . . . . . comma-shaped, simple curve, generally polar flagella.	*comma* *deneke* *finkleri*	

CLASSIFICATION OF MIGULA (MODIFIED)—*Continued*

Order.	Family.	Genus.	Species.
I. EUBACTERIA (true bact.) Suborder. A. *Haplobacterinæ* (lower bacteria).	*Spirillaceæ* curved (comma) or spiral forms.	*Spirillum*...... cork screw, several turns, non-flexible spiral, polar flagella.	*rubrum*
		Spirocheta..... flexible spirals, motile, no flagella.	*obermeieri*

See pp. 12 and 109–115, Marshall's Microbiology (1917).

Order.	Family.	Genus.
I. EUBACTERIA (true bacteria). Suborder B. *Trichobacterinæ* (higher bacteria).	*Chlamydobacteriaceæ* cylindrical cells in threads, ensheathed; reproduction by motile and non-motile gonidia.	*Chlamydothrix* unbranched threads, uniform in diameter.
		Crenothrix unbranched threads, filaments enlarged at free end.
		Phragmidiothrix branched and unbranched filaments. Cell division in 3 planes.
		Cladothrix dichotomous branching, uniform diameter.
II. THIOBACTERIA (sulphur bacteria).	*Beggiatoaceæ* cells contain sulphur granules.	*Thiothrix.* threads, non-motile and attached; sheath; gonidia.
		Beggiatoa no sheath, flat cells, motile with undulating membrane; no gonidia.
	Rhodobacteriaceæ cells contain bacteriopurpurin, sometimes sulphur granules. 5 sub-families.	1 *Thiocystis*, *Thiocapsa*, *Thiosarcina*
		2 *Lamprocystis*
		3 *Thiopedia*
		4 *Amœbobacter*, *Thiothece*, *Thiodictyon*
		5 *Chromatium*, *Rhabdochromatium*, *Thiospirillum*

NOTE.—See Marshall's Microbiology, 3rd Ed., 1921, pp. 114–117, for classification proposed by the Society of American Bacteriologists.

SPECIAL MEDIA

Litmus lactose agar for demonstrating acid production of microorganisms: Prepared the same as ordinary nutrient agar (see Exercise 9, Part I), with the exception that 1% lactose and 2% of the standard azolitmin solution is added just after filtration, while the agar is still hot, and well mixed through the agar before tubing. Sterilize by Tyndall method.

Dextrose calcium-carbonate agar for showing acid formation by microorganisms: Prepared the same as ordinary nutrient agar, with the exception that 1% dextrose and 1% $CaCO_3$ are added to the hot agar just after filtration. The added chemicals must be mixed well through the agar and care must be taken during tubing that the $CaCO_3$ remains in homogeneous suspension throughout the medium. Sterilize by discontinuous method.

Sour whey for determining the acid-destroying power of microorganisms: Inoculate sweet milk with a pure active culture of *Bact. lactis acidi* or *Bact. bulgaricum* as desired, and place at about 30° C. Allow the maximum acidity to form, cut the curd and heat in flowing steam for twenty or thirty minutes. Strain through clean cheese-cloth and allow to drain. Filter through filter paper. If clear whey is desired, it will be necessary to clear the medium with egg albumin.

Butter fat for demonstrating fat decomposition: Melt butter at about 100° C. and allow the casein to settle. Decant the clear fat, place about 8 c.c. in sterile test tubes and sterilize by the intermittent method.

Other kinds of fat may be prepared similarly.

Fermented agar for making solid synthetic media and for testing food requirements and selective powers of bacteria: 1. Place a weighed amount (three parts) of agar in a large bottle and to this add 200 parts of distilled water.

2. Cover the mouth of the bottle with parchment paper

or several layers of clean cheese-cloth and allow to ferment spontaneously.

3. Change the water in the bottle occasionally, replacing the amount of water removed, with the same amount of clean, distilled water.

4. When the active fermentation (as noted by the evolution of gas) has ceased entirely, this agar should be placed in an agateware pail, counterpoised, boiled over a free flame to dissolve the agar, counterpoised and any loss made up with distilled water.

5. Place in tubes or flasks as desired and autoclav.

Brilliant green agar for typhoid isolation: A clear 1.5% agar is essential; reaction neutral to Andrade's indicator, or +0.6%, +0.7% normal to phenolphthalein. For convenience make slightly alkaline to litmus and adjust to proper reaction when ready to use. Store agar in 100 c.c. quantities.

When ready to use, adjust and add to each 100 c.c. of agar base the following:

Andrade's indicator	1%
Lactose	1%
Dextrose	0.1%

Brilliant Green 0.1% aqueous solution 0.2 c.c. or 0.3 c.c.

Brilliant green solution is made as follows: weigh 0.1 gram of dye (samples from Bayer or Grübler and Höchst have proved satisfactory) accurately on a foil. Wash with boiling water into a 100 c.c. volumetric flask and make up to mark when cool.

Standardization. The reaction of the agar is important. It should be as clear as possible; sediment reduces activity of dye and light colored media are best. No two lots of agar act the same; a new batch of agar or a new solution must be tested. Any variation in the composition of media necessitates a readjustment of dye concentration. It is important

to bear this in mind. Solutions keep about a month and should be discarded after that time.

Brilliant green not only differentiates colon-typhoid organisms but also inhibits, in appropriate dilutions, all Gram-positive and many Gram-negative bacteria. *B. paratyphosus* and *B. lactis aerogenes* are unaffected, *B. typhosus* is restrained only at low dilutions, while *Bact. dysenteriæ* and the other members of the colon are extremely susceptible. The typhoid colony is characteristic of this medium. The colony is large and has a snowflake appearance when held against a dark surface in oblique light. The edge is delicately serrate. The texture is that of a coarse woolen fabric with artificial light when examined by a hand lens.

Loeffler's medium: Collect beef or horse blood in sterile vessels, preferably high cylindrical jars. Every precaution should be exercised in the collection of blood, such as keeping vessels covered before and after collecting; allowing first blood to flow without collecting so as to wash out contaminating material. This is especially true when blood is collected while animals are being slaughtered.

Allow blood to coagulate before removing from slaughter house, otherwise it is liable to be cloudy. Separate adhesions between the clot and jar with a sterile glass rod or wire. Place in ice-chest for twenty-four hours. Remove clear supernatant serum with a large pipette 50 to 100 c.c. capacity.

To three parts of the serum add one part of a 1% dextrose veal or beef infusion broth. Fill small test tubes with this mixture and inspissate. The best results are obtained by placing the tubes for a short time in hot water at about 50°, not above, to drive out air. Unless good judgment is exercised the medium will be broken up by air bubbles. Place in inspissator and raise temperature very gradually to 75° and maintain for three hours. Repeat this process for five successive days. Place water or damp cloth in inspissator to keep media from drying out. A quicker method is to

place in the Arnold sterilizer after the first and second inspissation and bring the temperature up very gradually.

Dorset's egg medium: This medium is used for the cultivation of tubercle bacilli.

Thoroughly clean four eggs with water, wash in 5% phenol and allow to partially dry. Dry ends of eggs in flame and pierce shell with a pair of sterile forceps, breaking the membrane in one end and leaving it intact in the other. By blowing through the latter opening the contents of the eggs are expelled. Collect in a sterile Erlenmeyer flask.

Break up yolk with a sterile platinum wire and thoroughly mix the whites and yolks.

Add 25 c.c. of distilled water and strain through sterile cloth. Fill sterile test tubes with about 10 c.c. of the medium and stand in water at 45° C. for ten to fifteen minutes to drive out air. Slant in an inspissator and expose to 70°–75° C. for four or five hours on two successive days. Gradually raise temperature above this on third day and finish sterilization by a single exposure to 100° C. in an Arnold sterilizer. Before inoculation add two or three drops of sterile broth to each tube.

If human tubercle bacilli are to be cultivated, add 5% glycerin to medium.

Petroff's medium: This medium consists of two parts of egg (white and yolk), one part of meat juice and gentian violet in the proportion of 1 : 10,000; 500 grams of beef or veal are infused in 500 c.c. of a 15% solution of glycerin in water. After twenty-four hours' infusion the meat is squeezed in a sterile meat press and collected in a sterile beaker. Sterilize the shells of the eggs by immersion for ten minutes in 70% alcohol or by pouring hot water upon them. Break the eggs in a sterile beaker and after mixing the eggs well with a sterile platinum needle, filter through sterile gauze. Add one part by volume of meat juice. Add sufficient 1% alcoholic gentian violet to make a dilution of 1 : 10,000.

Tube about 3 c.c. in each sterile test tube and inspissate for three successive days; on the first day at 85° C., until all the media is solidified. Sometimes it is necessary to change the places of the tubes. On the second and third days at 75° C. for not more than an hour. The glycerin is omitted for the bovine type, although it grows if glycerin is present.

Uschinsky's asparagin medium: protein-free.

Asparagin, $COOH \cdot CH(NH_2) \cdot CH_2 \cdot CO \cdot NH_2$..	3.4 gms.
Sodium chloride, $NaCl$......................	5.0 gms.
Magnesium sulphate, $MgSO_4$..............	0.2 gm.
Calcium chloride, $CaCl_2$....................	0.1 gm.
Monobasic acid potassium phosphate, KH_2PO_4.	1.0 gm.
Iron sulphate, $FeSO_4$........................	Trace
Distilled water..............................	1000.0 c.c.

Cohn's solution: inorganic nitrogen combined with an organic acid.

Monobasic acid potassium phosphate, KH_2PO_4.	5.0 gms.
Calcium phosphate, Ca_3PO_4................	0.5 gm.
Magnesium sulphate, $MgSO_4$..............	5.0 gms.
Ammonium tartrate, $\begin{array}{l} CH(OH) \cdot COO \cdot NH_4 \\ \vert \\ CH(OH) \cdot COO \cdot NH_4 \end{array}$....	10.0 gms.
Distilled water..............................	1000.0 c.c.

Winogradski's medium for nitrate formation: inorganic nitrogen combined with inorganic acid.

Ammonium sulphate, $(NH_4)_2SO_4$............	0.40 gm.
Magnesium sulphate, $MgSO_4$...............	0.05 gm.
Dibasic acid potassium phosphate, K_2HPO_4..	0.10 gm.
Sodium carbonate, Na_2CO_3.................	0.60 gm.
Calcium chloride, $CaCl_2$....................	Trace
Distilled water............................	1000.0 c.c.

Winogradski's medium for symbiotic nitrogen-fixation: nitrogen-free.

Dibasic acid potassium phosphate, K_2HPO_4..	1.00 gm.
Magnesium sulphate, $MgSO_4$..............	0.50 gm.
Sodium chloride, NaCl....................	0.01 gm.
Ferric sulphate, $Fe_2(SO_4)_3$.................	0.01 gm.
Manganese sulphate, $MnSO_4$...............	0.01 gm.
Dextrose, $CH_2OH(CHOH)_4CHO$...........	20.00 gms.
Distilled water..........................	1000.00 c.c.

Gelatin for cultivating phosphorescent halophilic organisms: Prepared as ordinary gelatin with the addition of 3% salt. The reaction is made −2.0%.

Fermented cider for the cultivation of acetic bacteria: Inoculate unfermented cider with *Sacch. ellipsoideus* and allow to proceed until the evolution of gas ceases. Filter, place in tubes and flasks as desired. Pasteurize.

MEDIA FOR SOIL MICROBIOLOGY.

Soil extract: 1. Boil 1 kg. of good rich garden soil with 2 liters of tap water for two hours over the free flame.

2. Pour off the turbid liquid, mix some talc and filter through a double filter paper. If the first filtrate is turbid refilter through the same paper.

3. Make up to 800 c.c. with tap water.

4. Place in tubes or flasks as desired and autoclav.

Soil extract agar is prepared by adding 1.5% washed agar to the soil extract prepared as above.

Soil may be plated either in soil extract agar (or other special agar) or in ordinary agar, gelatin, etc. On account of the diversity of the requirements of the various species of microorganisms in soil, no one medium will suffice for the cultivation of all species. Emphasis is therefore not laid on any particular medium for plating soils.

Starch Agar:

A. Agar, washed	10 gms.
Dibasic potassium phosphate K_2HPO_4	1 gm.
Magnesium sulfate $MgSO_4+7H_2O$	1 gm.
Sodium chloride NaCl	1 gm.
Ammonium sulfate $(NH_4)_2SO_4$	2 gms.
Calcium carbonate $CaCO_3$	2 gms.
Tap water	500 c.c.

Counterpoise digest agar over free flame in water containing the above salts, counterpoise and make up loss in weight with boiling water. To this add:

B. Starch solution.

Make a smooth paste of 10 gms. of potato or other starch in a little cold water. Add 800 c.c. boiling water. Concentrate to 500 c.c. by boiling. This breaks up the starch grains and gives a nearly transparent solution.

Boil *A* and *B* together a few minutes to obtain a homogeneous mixture, then strain through two thicknesses of cheese cloth. This removes large particles of foreign matter yet leaves the insoluble $CaCO_3$ in suspension. Add 1.5% China blue—rosolic acid for indicator. Care must be exercised in tubing and after sterilization in the use of this and other similar solid media that the insoluble material be kept in homogeneous suspension while tubing and inoculating respectively.

Starch agar: Add 10% of corn starch to beef-infusion (not extract) agar prepared in the usual way except salt and peptone are omitted. Adjust to an acidity of 0.2 to 0.5% normal. Sterilize in autoclav at ten pounds for thirty minutes.

This medium is good for the cultivation of gonococcus and meningococcus.

Blood media: If the serum of blood is desired, it may be collected as described in the preparation of Loeffler's medium. There are several methods for the sterilization of serum.

One method is to expose the serum, after it has been pipetted in test tubes, to temperatures ranging from 60° to 65° C. for one hour on six successive days. Serum may also be sterilized by filtration through a Berkefeld or Pasteur-Chamberland filter. This method is very effective but requires care and much time.

The best way is to collect the blood in sterile flasks or test tubes using a sterile canula. Allow to clot and pipette off the serum. Exudate or transudate fluids may also be collected in this way from the pleural cavity, the hydrocele cavity or the abdominal cavity.

Whole blood may be obtained from rabbits, dogs or other accessible animals by bleeding directly from a blood vessel into melted agar.

Agar used for this purpose is melted and cooled to 60°C. or lower. Then the whole blood, blood serum, or other body fluid is added. About 1% of whole blood makes a satisfactory medium. Blood serum or other body fluids are added in the proportion of 3:1, i.e. three parts agar and one part fluid.

Conn's asparaginate agar: These two media used for the determination of numbers of microorganisms in soil permit the development of much larger numbers than most other solid nutrient media, synthetic or otherwise.

Sodium asparaginate, $COONa \cdot CH_2 \cdot CHNH_2 \cdot COOH + H_2O$	1.0 gm.
Dextrose $C_6H_{12}O_6$	1.0 gm.
Magnesium sulfate $MgSO_4$	0.2 gm.
Monobasic ammonium phosphate, $NH_4H_2PO_4$	1.5 gms.
Calcium chloride, $CaCl_2$	0.1 gm.
Potassium chloride, KCl	0.1 gm.
Ferric chloride, $FeCl_3$	trace
Agar	12.0 gms.
Distilled water	1000.0 c.c.

Adjust the reaction between +0.8 and +1.0% normal to phenolphthalein. *This is very important.*

From Tech. Bul. 38 (1914), N. Y. (Geneva) Agr. Expt. Sta., p. 9.

Brown's albumen agar:

Dibasic potassium phosphate, K_2HPO_4,	0.5 gm.
Magnesium sulfate, $MgSO_4$	0.2 gm.
Powdered egg albumen	0.1 gm.
Dextrose, $C_6H_{12}O_6$	10.0 gms.
Ferric sulfate, $Fe_2(SO_4)_3$	trace
Agar	15.0 gms.
Distilled water	1000.0 c.c.

This medium must not be filtered.

From Res. Bul. 11 (1913) Iowa Agr. Expt. Sta.

Conn's agar for actinomycetes:

Agar	15.0 gms.
Glycerin	10.0 gms.
Sodium asparaginate, ($COONa \cdot CH_2CHNH_2COOH + H_2O$)	1.0 gm.
Glucose ($C_6H_{12}O_6$)	1.0 gm.
Ammonium hydrogen phosphate, $NH_4H_2PO_4$	1.5 gms.
Magnesium sulfate ($MgSO_4 + 7H_2O$)	0.2 gm.
Calcium chloride ($CaCl_2$) fused	0.1 gm.
Potassium chloride (KCl)	0.1 gm.
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	1000.0 c.c.

From Jour. Bact. Vol. 1, p. 198 (1916).

Omeliansky's medium for anaerobic cellulose fermentation:

Filter paper (in strips). Cotton, straw, or starch may be substituted for filter paper	2.0 gms.

$CaCO_3$	20.0 gms.
K_2HPO_4	1.0 gm.
$MgSO_4$	0.5 gm.
$(NH_4)_2SO_4$	1.0 gm.
NaCl	Trace
Distilled water	1000.0 c.c.

Method. 1. Introduce substances in order named into 1000 c.c. distilled water.

2. Stir to dissolve all soluble substances and tube while insoluble substances are in homogeneous suspension, placing about 10 c.c. in each tube.

3. Sterilize in autoclav.

Media for studying urea decomposition: Urea broth, gelatin and agar are generally prepared by adding 1% to 2% urea to the ordinary media. This medium favors the growth of *B. coli*, *B. proteus*, *B. erythrogenes*, etc.

Ordinary media to which 10% urea has been added favors the growth of *B. pasteurii*, a spore-producing bacterium.

Urea gelatin and agar may be prepared by adding 1 c.c. of a 15% aqueous solution of urea to each tube of the ordinary media after sterilization, and then heating the tubes again. This is the method preferred because the addition of urea reduces the solidifying power of the gelatin. A small amount of urea is converted into ammonia by heating in the steam, but this has little influence on the results obtained in the experiment. Heating in the autoclav is to be avoided!

Albuminoid-free culture solutions for studying urea decomposition:

I. Soil extract .... 100 c.c.
K_2HPO_4 .... 0.05 gm.
Urea .... 5.00 gms.

II. *Sohngen's solution.*
Tap water .... 100.00 c.c.

Urea	5.00 gms.
K_2HPO_4	0.05 gm.
Ammonium or calcium malate, or, calcium citrate or tartrate.	0.50 to 1.00 gm.

B. pasteurii will not grow in these solutions as it requires the presence of albuminoids in the medium.

Solutions for cultivating nitrifying bacteria:

I.	Distilled water	1000.0 c.c.
	$(NH_4)_2SO_4$	1.0 gm.
	K_2HPO_4	1.0 gm.
	$MgSO_4$	0.5 gm.
	NaCl	2.0 gms.
	$FeSO_4$	0.4 gm.

Add basic $MgCO_3$ after sterilizing.

This solution is adapted for relatively increasing the nitrite bacteria.

II.	Distilled water	1000.0 c.c.
	$NaNO_2$	1.0 gm.
	K_2HPO_4	0.5 gm.
	$MgSO_4$	0.3 gm.
	NaCl	0.5 gm.
	Na_2CO_3	0.3 gm.

This solution causes a greater relative increase in the nitrate producers.

III. The same as solution **I**, but instead of $MgCO_3$ $CaCO_3$ is added after sterilizing. This solution stimulates the simultaneous growth of both organisms, as in nature.

Culture solutions for denitrification studies. Nitrate broth or agar. Add 1 c.c. of a 1% solution of sodium or potassium nitrate to tubes of ordinary broth or agar (melted), mix well and re-sterilize.

Giltay's solution.

KH_2PO_4	2.0 gms.
$MgSO_4$	2.0 gms.
KNO_3	1.0 gm.
$CaCl_2$	0.2 gm.
Fe_2Cl_6 solution	2.0 drops
Citric acid	5.0 gms.

Method. 1. Dissolve the above substances in 800 c.c. of distilled water (solution **I**).

2. Add a few drops of phenolphthalein and, using a pipette, drop in just enough 10% NaOH to turn the solution a faint pink.

3. Dissolve 10 gms. dextrose in 200 c.c. of distilled water (solution **II**).

4. Mix solutions **I** and **II** very thoroughly.

5. Sterilize in the autoclav at 15 lbs. pressure for ten minutes. (Lipman and Brown.)

Giltay's agar is prepared by adding 1.5% washed agar to the above solution. Boil until dissolved. Filter through absorbent cotton. Sterilize in autoclav.

Mannit solution for nitrogen-fixing organisms.

Mannit	15.0 gms.
K_2HPO_4	0.2 gm.
$MgSO_4$	0.2 gm.
NaCl	0.2 gm.
$CaSO_4$	0.1 gm.
$CaCO_3$	5.0 gms.
10% Fe_2Cl_6 solution	1.0 drop

Method. 1. Add the above chemicals to 1000 c.c. distilled water.

2. Titrate using phenolphthalein and neutralize using normal NaOH.

Do not filter. The presence of $CaCO_3$ offers an additional means of isolating *Azotobacter*, as these organisms are found in soil in much greater numbers around the particles of calcium carbonate.

3. Sterilize at 120° C. (autoclav), for ten minutes.

Mannit agar is prepared by adding 1.5% washed agar to the above solution, boiling until the agar is wholly dissolved and sterilizing as above. *Do not filter.*

Nitrogen-free ash agar for cultivation of Ps. radicicola.

1. Stir 5 gms. of wood ashes (beech, elm, maple) into 1000 c.c. distilled water for two to three minutes *only.* Filter.
2. Add 1% washed agar.
3. Heat in steam for thirty minutes.
4. Then add 1% commercial saccharose.
5. Boil five minutes over a free flame.
6. Strain while hot through several thicknesses of clean cheese-cloth. This may be filtered if desired.
7. For Exercise 9, Soil Microbiology, tube, placing about 6 cm. of agar in the large test tubes with foot, the rest in ordinary test tubes. Sterilize. (Tyndall method.)

Nitrogen-free solution may be prepared as above, omitting the agar.

Congo-red agar for differentiating Ps. radicicola from *Bact. tumefaciens:*

Distilled water	1000.00 c.c.
Saccharose	10.0 gms.
K_2HPO_4	1.0 gm.
$MgSO_4$	0.2 gm.
Washed agar	15.0 gms.
Congo-red	0.1 gm.

Solution for sulphate reduction:

Tap water	1000.0 c.c.
K_2HPO_4	0.5 gm.
Sodium lactate	5.0 gms.
Asparagin	1.0 gm.
$MgSO_4$	1.0 gm.

A few drops of $FeSO_4$ solution. Sterilize in the autoclav.

WATER ANALYSIS MEDIA

Culture media for standard bacteriological water analysis must contain ingredients of a special nature.

Ingredients. 1. Distilled water in place of tap water.

2. Infusion of fresh lean meat instead of meat extract.

3. Witte's peptone (dry, from meat).

4. No salt.

5. Gelatin of the best French brand and as free as possible from acids and other impurities.

6. Commercial agar of as high a grade of purity as possible. Agar may be purified by washing.

7. Dextrose, lactose, saccharose, etc., of sugar media, chemically pure.

8. A 1% aqueous solution of Kahlbaum's azolitmin may be used in place of litmus.

Sterilization. Sterilize media in the autoclav at 120° C. (15 lbs. pressure) for fifteen minutes. A shorter period than this may result in incomplete sterilization, a longer period will probably result in inversion and caramelization of the sugars and in lowering the melting-point of the gelatin. *Have the sterilizer hot when the medium is inserted so that heating to the point of sterilization will be accomplished as quickly as possible; cool rapidly upon removing from the autoclav.*

The Tyndall (intermittent) method may be employed, heating for thirty minutes on three successive days.

Reaction. Phenolphthalein is used as indicator.

Titrate media while hot with N/20 NaOH and adjust the reaction if necessary. All media should have a +10° reaction Fuller's scale unless otherwise stated in the directions.

Distribution of Work. It may be desirable to have students work in groups in preparing media. The following plan has worked satisfactorily:

Students may work in groups of five, one of the groups

preparing a sufficient quantity of medium for himself and the other four members of the group, dividing the work up as follows:

One student prepare agar shakes and litmus milk.

One student prepare gelatin.

One student prepare litmus lactose agar.

One student prepare litmus lactose bile.

One student prepare Dunham's solution and nitrate peptone solution.

In this arrangement each student must furnish the respective sterile glassware sufficient for containing the various necessary media, to the student preparing each medium.

Each student of the group *must so plan his work* that the medium he prepares will be *finished*, *sterilized* and *ready for use at the same time* as those of the remaining members of his group.

Media. *Litmus lactose agar shake.*

I. 2% washed agar.
2% peptone.
2% lactose.
2% azolitmin.
500 c.c. meat infusion.
500 c.c. distilled water.

Method. **1.** Strain the meat infusion through a piece of clean cheese-cloth.

2. Place the washed agar in the distilled water, weigh, digest over a free flame, weigh again and make up any loss with distilled water.

3. To the hot agar add the peptone and lactose and mix until dissolved; then add the strained meat infusion.

4. Titrate and adjust the reaction to 0°.

5. Add the azolitmin, boil up over the free flame and place about 100 c.c. in sterile 250 c.c. Florence flasks.

Each student will need four litmus lactose agar shakes.

II. *Litmus lactose agar.* (To be used in tubes for plating only.)

1.5% agar.
1.0% peptone.
1.0% lactose.
2.0% azolitmin solution.
500 c.c. meat infusion.
500 c.c. distilled water.

Method. **1.** This agar is prepared as ordinary nutrient agar making the reaction +1%, *adding the lactose and azolitmin just before tubing.*

2. Tube and sterilize by the Tyndall method.

Each student will need at least forty tubes of litmus lactose agar.

III. *Gelatin.*

15% gelatin.
1% peptone.
500 c.c. meat infusion.
500 c.c. distilled water.

Method. Prepare, tube and sterilize as for ordinary gelatin.

Salt is omitted. Reaction +1%.

Each student will need forty or fifty tubes of salt-free gelatin.

IV. *Sugar-free broths and sugar broths.* (Neutral red dextrose broth.)

Method. **1.** Heavily inoculate a tube of sterile broth with *B. coli* and incubate at 37° C.

2. Soak 1 lb. finely chopped lean beef in 1000 c.c. distilled water over night (twenty-four hours).

3. Strain out the meat juice and make up to 1000 c.c. with distilled water.

4. Pour the entire contents of the twenty-four-hour broth culture of *B. coli* into the meat juice and

5. Incubate at 37° C. for twelve to sixteen hours, *not*

longer. *B. coli* uses the fermentable substances, inosite (muscle sugar), dextrose, etc., as food, leaving the meat juice free from fermentable substances. *If this action is allowed to proceed too long, poisonous decomposition products of the proteins are formed which will inhibit the growth of other microorganisms.*

6. Mix the peptone (1%) into a thin paste with as little water as possible and add to the twelve or sixteen-hour culture of *B. coli* in the meat juice.

7. Heat in the autoclav for twenty minutes or in the steam for one hour.

8. Titrate and make neutral to phenolphthalein.

9. Boil over a free flame for three to five minutes.

10. Add 1% dextrose and 10 c.c. of 0.5% solution of neutral red and stir until sugar is dissolved.

11. Filter until clear.

12. *Fill ten fermentation tubes for each student.*

13. Sterilize in autoclav or in flowing steam.

14. Other sugar broths are prepared by adding instead of dextrose, 1% of the sugar desired.

Practically *all* sugar-fermenting organisms will ferment monosaccharides such as dextrose; comparatively few will ferment the disaccharides lactose, saccharose, etc. *B. coli* will ferment all three sugars to a greater or less extent. Bacteria of the typhoid group ferment *none* of the three and those belonging to the paratyphoid group ferment dextrose but *not* lactose, therefore the use of lactose in culture media will inhibit to a great extent the growth of the last two groups and favor the development of the organisms of the *B. coli* group. This group is by far the largest, occurs most often and in greatest numbers in sewage and like material, therefore tests for this group are used as indication of the presence of intestinal organisms in the material (water in this case) to be examined.

V. *Litmus lactose bile salt medium.*

Bile salts are invaluable for certain media used for

water analysis as they inhibit organisms of practically all but the intestinal type.

20 gms. peptone.
5 gms. sodium taurocholate.
10 gms. lactose.
20 c.c. 2% azolitmin solution.
1000 c.c. distilled water.

Method. 1. Dissolve the bile salt and peptone in the water and boil.

2. Add the lactose and sufficient azolitmin to give a distinct purple tint.

3. Filter, fill into fermentation tubes and sterilize by intermittent method.

Each student needs four litmus lactose bile salt fermentation tubes.

VI. *Esculin bile solution* for *B. coli* test.

10.0 gms. peptone.
5.0 gms. sodium taurocholate.
0.1 gm. esculin.
0.5 gms. soluble iron citrate.
1000.0 c.c. distilled water.

Method. 1. Dissolve the ingredients in the order given, clear with egg albumen, tube and sterilize (see Prescott and Winslow's Elements of Water Bacteriology, 3d Ed., p. 279). This solution has a blue fluorescence.

VII. *Dunham's solution;* twenty-five tubes for each student. (See p. 42).

VIII. *Nitrate, peptone solution;* twenty-five tubes for each student. (See p. 44.)

IX. *Litmus milk;* twenty-five tubes for each student. (See p. 24.)

X. *Hiss serum-water media for fermentation tests:* This medium is especially adapted to test the sugar splitting and the acid producing powers of various bacteria.

Method. Dilute beef or sheep serum obtained from clotted blood with three times its volume of distilled water. Heat mixture in the Arnold for fifteen minutes. Divide into desired quantities and add 1% of the sugar to be used and enough litmus to give a deep purple color. Sterilize by the fractional method.

XI. *Adonite broth:* To make adonite broth use plain broth and add 0.5% adonite. *B. aerogenes* and *B. cloacæ* ferment adonite.

XII. *Andrade indicator.*

Method. Make a 0.5% aqueous solution of acid fuchsin. To 100 c.c. of the acid fuchsin solution add 16 c.c. of normal sodium hydroxide solution. The alkali changes the red of the fuchsin to orange or yellow. Allow to stand about twelve to fourteen hours before using. Filter if necessary.

XIII. *Methylene blue-eosin medium (Holt, Harris and Teague).*

Method. Add 0.5% lactose, 0.5% saccharose, and 2 c.c. of a 2% solution of yellowish eosin to 100 c.c. of melted stock agar and mix well. Then, add 2 c.c. of a 0.5% solution of methylene blue to the above and mix thoroughly. Pour plates before agar cools. The solutions of dyes should be added separately and thoroughly mixed because the protective colloidal action of the agar prevents the combination and precipitation of the two dyes. When acid is produced by the fermenting types of microorganisms this protective action is destroyed and colored colonies result. If the colonies are too numerous, the color change is impeded. The dye solutions should be prepared in distilled water and kept in the dark.

XIV. *Methyl red medium:* **Method. 1.** To 800 c.c. distilled water add 5 grams Witte's peptone (other peptones should not be substituted) 5 grams C.P. dextrose, and 5 grams dipotassium hydrogen phosphate (K_2HPO_4). A dilute solution of the K_2HPO_4 should give a distinct pink with phenolphthalein.

2. Heat with occasional stirring over steam for twenty minutes.

3. Filter through folded filter paper, cool to 20° C. and dilute to 1000 c.c. with distilled water.

4. Distribute 10 c.c. portions in sterile test tubes.

5. Sterilize by intermittent method for twenty minutes on three successive days.

XV. *Methyl red indicator:* Dissolve 0.1 gram methyl red in 300 c.c. alcohol and dilute to 500 c.c. with distilled water.

XVI. *Methyl red test:* After the cultures have grown for five days in dextrose broth, take 5 c.c. of the broth and add five drops of the methyl red indicator. A distinct red color is positive, a distinct yellow color is negative, and intermediate colors are doubtful.

XVII. *Voges-Proskauer test*: After the cultures have grown for five days in dextrose broth, take 5 c.c. of the medium, add 5 c.c. of a 10% solution of potassium hydroxide. Allow to stand over night. An eosin pink color indicates a positive test.

XVIII. *Tryptophane broth for Indol test*: To 1000 c.c. of distilled water add 0.3 gram tryptophane, 5 grams di-potassium hydrogen phosphate (K_2HPO_4) and 1 gram peptone. Heat until ingredients are thoroughly dissolved, tube (about 8 c.c. to tube) and sterilize in autoclav for fifteen minutes at fifteen pounds pressure.

XIX. *Standard Endo media for water analysis:* The Standard methods Endo medium is made as follows: 3% agar base is made neutral to phenolphthalein, flasked, sterilized, and stored in convenient quantities (usually 200 c.c.). When ready to use, add 2 grams of lactose to 200 c.c. of the agar and melt in steam. Prepare a 10% solution of anhydrous sodium sulphite and to 10 c.c. of this solution add 2 c.c. of a 10% solution of basic fuchsin in 95% alcohol, heat for a few minutes. To the 200 c.c. of melted lactose agar add 1 c.c. of the above fuchsin-sulphite solution. Pour plates, allow to harden, and place in incubator to dry.

The *Hygenic Laboratory-Endo medium* is made as follows: 3% agar base made +0.5% to phenolphthalein. Add 3.7 c.c. of a 10% solution of anhydrous sodium carbonate. Place in 200 c.c. quantities in Erlenmeyer flasks, sterilize and store until used. When ready to use, dissolve the agar and add the following ingredients to each 200 c.c. of agar. Dissolve 2 grams of lactose in 25 to 30 c.c. of distilled water. Dissolve 0.5 gram of anhydrous sodium sulphite in 10 to 15 c.c. of distilled water. To the sulphite solution add 1 c.c. of saturated solution of basic fuchsin in 95% alcohol. Add the fuchsin-sulphite solution to the lactose solution and then add the whole to the agar. Pour plates at once, allow to harden, and dry for fifteen minutes in the incubator.

XX. *Russell's double sugar agar:* A stiff agar (2 to 3%) is used. Adjust to about +0.7% normal. Add litmus until a distinct purple-violet color is produced. It may be necessary to add more alkali to get the desired color. One per cent. lactose and 0.1% dextrose is then added. Sterilize preferably by Tyndall's method. Make a medium slant and a deep bottom to the agar in the tube after it is sterilized. If Andrade's indicator is available, use it in place of litmus.

The reactions for the colon-typhoid group when litmus is used is as follows: Typhoid shows a delicate growth on the violet slant and a deep pink in the butt of the tube. The paratyphoids show gas bubbles in a pink butt with violet slant. Paratyphoid B. usually shows more gas than paratyphoid A. The colon organism turns both slant and butt red with production of gas in the butt. The dysentery organisms show a growth on the violet slant and a deep pink in the butt of the tube.

XXI. *Krumwiede's triple sugar agar:* Three per cent. agar is desirable. Adjust reaction to +0.2% normal. Add 1.0% lactose, 1.0% saccharose, 0.1% dextrose, and 1% Andrade's indicator or 2% litmus. Tube and sterilize. Slant tubes so that the agar forms a deep butt and a medium slant.

Inoculate by stabbing the butt and streaking the slant. With Andrade's indicator, typhoid gives an acid butt without gas and a clear top. Paratyphoids give acid butt with gas and clear top. The colon organisms give an acid butt with gas and a red top. The dysentery organisms give acid butt without gas and clear top.

The only difference between double and triple sugar agar is the addition of saccharose to the latter.

Many organisms give a characteristic reaction on Russell's double sugar medium which are not paratyphoids. They are "intermediates" and ferment lactose slowly or not at all. Many of these organisms are strong fermenters of saccharose. Hence the addition of saccharose. In this way approximately one-half of these troublesome forms may be eliminated, and the usefulness of the original medium is not impaired. Andrade's indicator is preferable to litmus in either medium.

XXII. *Lead acetate medium for paratyphoids:* Take 1.5% nutrient agar and add 1% dextrose, 1% lactose and 0.05% basic lead acetate. Make up a 0.5% solution of basic lead acetate and sterilize. Then add the required amount to make 0.05% to each tube of agar before sterilizing.

The basic lead acetate medium is useful in differentiating the paratyphoids. Paratyphoid B. and B. typhosus give a brownish discoloration while paratyphoid A. and B. dysenteriæ give no coloration.

XXIII. *Agar base for Endo's, Conradi-Drigalski, litmus lactose agar, etc., used in feces work:*

Liebig's extract	5 gms.
Salt	5 gms.
Peptone	10 gms.
Agar	30 gms.
Water to make	1,000 c.c.

Prepare as ordinary agar, and adjust reaction to 0 or +0.2. A stiff agar (3%) is used to check the diffusion of acid

beyond the colony and also to check spreading of colonies. Keep in 100 c.c. quantities in Erlenmeyer's flasks.

XXIV. *Endo's medium for feces, colon-typhoid, etc.:* Melt a flask of the above agar, and while liquid add to the 100 c.c. six drops of a saturated alcoholic solution of basic fuchsin (4 grams fuchsin to 100 c.c. of 95% alcohol). Mix well, then add about eighteen or twenty drops of a *freshly* prepared 10% solution of sodium sulphite. The sulphite solution will decolorize the intense red of the basic fuchsin to a light rose pink. As the medium cools, this color fades to a pale salmon. While the agar is still hot, add 5 c.c. of a freshly prepared hot aqueous 20% solution of chemically pure lactose.

XXV. *Conradi-Drigalski's medium.*

Agar Base

3 lbs. meat infused twenty-four hours in 2,000 c.c. water

Peptone	20 gms.
Nutrose	20 gms.
Sodium chloride	10 gms.
Agar	60 gms.

Make into nutrient agar in the usual way and render slightly alkaline to litmus paper.

Litmus solution: (Conradi and Drigalski used a very sensitive aqueous litmus recommended by Kubel and Tiemann). Very satisfactory results may be obtained by dissolving 2.5 grams of Kahlbaum's azolitmin in 100 c.c. of distilled water. Add 40 c.c. of this solution to the agar base.

Dissolve 30 grams of lactose in 100 c.c. of water and add to the hot agar solution. Adjust reaction to weakly alkaline with litmus paper.

To the resultant mixture, add 4 c.c. of a hot sterile 10% solution of sodium carbonate, and 20 c.c. of a freshly made solution of crystal violet. (0.1 gram crystal violet in 100 c.c. sterile distilled water).

Typhoid and dysentery are small transparent bluish-gray colonies. Colon colonies are larger, red and opaque.

EXPLANATION OF TABLE ON PAGES 389–390

Method. *B. coli*- and *B. choleræ suis*-like organisms: Place 5 c.c. of suspected water in each dextrose and liver broth fermentation tube and 50 to 100 c.c. in a litmus lactose agar flask. Incubate at 37° C. If gas appears in time of three days, make plating on Conrad-Drigalski's agar * from one showing most of gas production. Isolate different colonies on agar slants. From the growth on the agar slants inoculate different media to subgroup the organisms and consequently to identify them.

B. typhosus: Hoffman and Fiske enrichment medium. Add to the suspected water 1.0% of nutrose; 0.5% of caffein; 0.001% of crystal violet. Incubate at 37° C. for *not more than* twelve to thirteen hours. Make Endo or Conradi-Drigalski agar plates. Isolate bluish colonies, transferring to agar slant, and identify. The Widal reaction should be used for the confirmatory test.

(Data on pages 389–390 collected by O. M. Gruzit.)

* Other media for bacteriological water analyses will be found in the 1915 edition of the "Standard Methods for the Examination of Water and Sewage" published by the American Public Health Association, pp. 124–137.

This publication is the standard work; references to special phases will be found in the bibliography following each chapter.

IDENTIFICATION OF BACTERIA IN POLLUTED WATER

WATER

- Liver broth, gas. Dextrose broth, gas.
  - *B. coli* group. Lactose litmus agar shake, gas, acid.
  - *B. choleræ suis*-like organisms. Lactose litmus agar shake, no gas or acid.
    - Conradi-Drigalski's Agar.
      - Red colonies: Dextrose +, Lactose +
        - Dulcit +, Adonit −, Gelatin −: *B. coli communis*, *B. coli communior*, *B. aerogenes* 2.
          - Saccharose +: *B. coli communior*, *B. aerogenes* 2.
            - Indol +, Esculin bile + (8 days), NaOH red react. −: *B. aerogenes* 2. Motility +
            - Indol ±, Esculin bile −, NaOH red react. +: *B. coli communior* (Emmerich) Motility −
          - Saccharose −, Motility +: *B. coli communis* (Escherich).
        - Dulcit −: *B. cloacæ*. *B. acidi-lactici*, *B. lactis aerogenes*, *B. aerogenes* 1. *Bact. aerogenes*.
          - Adonit −, Gelatin +, Saccharose +, Motility +: *B. cloacæ* (Jordan).
          - Adonit +, Gelatin −, Motility −: *B. lactis aerogenes*, *B. acidi-lactici*, *B. aerogenes* 1 and *Bact. aerogenes*.
            - Saccharose −, Indol +, Nitrate red +, Esculin bile −: *B. acidi-lactici* (Hueppe).
            - Saccharose +, Indol −, Nitrate red +, Esculin bile −: *Bact. aerogenes* (Pfeiffer).
            - Saccharose +, Indol ±, Nitrate red +, Esculin bile + (1 da): *B. lactis aerogenes* (Escherich).
            - Saccharose +, Indol +, Nitrate red +, Esculin bile + (3 da). *B. aerogenes* 1.
      - Blue colonies: Dextrose +, Lactose −: *B. paratyphosus* A. *B. paratyphosus* B. *B. choleræ suis*.
        - Dulcit −, Motility +, Lit. milk alk.: *B. choleræ suis*
        - Dulcit +, Motility +: *B. paratyphosus* A. *B. paratyphosus* B.
          - Lit. milk acid. *B. paratyphosus* A.
          - Lit. milk at first acid, later alk. *B. paratyphosus* B.
- Hoffman and Fiske enrichment medium 12 to 13 hours. Endo or Conradi-Drigalski's agar plates.
  - Blue colonies: Dextrose −, Lactose −: *B. typhosus*

FERMENTATIVE PROPERTIES OF SOME COMMON BACTERIA

		Dextrose.	Lactose.	Dulcite.	Saccharose.	Mannite.	Raffinose.	Levulose.	Galactose.	Arabinose.	Maltose.	Sorbite.	Salicin.	Inulin.	Dextrin.	Litmus milk.
B. proteus	Acd	Acd	0	0	Acd	0	0	0	0	Acd	Acd	0	0	0	0	
	Gas	+	–	–	+	–	–	–	–	+	+	–	–	–	–	
B. fæcalis alkaligines	Acd	Alk	Alk		Alk	Alk		Alk	Alk		Alk				Alk	Alk
	Gas	–	–		–	–		–	–		–				–	–
Bact. dysenteriæ Shiga	Acd	Acd	Alk		Alk	Alk	Acd	Acd	Acd	Acd	Alk	0		0	Alk	Acd
	Gas	–	–		–	–	–	–	–	–	–	–		–	–	Alk
Bact. dysenteriæ Flexner	Acd	Acd	Alk		Acd	Acd	Acd	Acd	Acd	Acd	Acd	Acd*	Acd*	Acd*	Acd	Acd
	Gas	–	–		–	–	–	–	–	–	–	–	–	–	–	Alk
Bact. dysenteriæ "Y"	Acd	Acd	Alk		Alk	Acd		Acd	Acd		Alk				Alk	Acd
	Gas	–	–		–	–		–	–		–				–	Alk
Bact. diphtheriæ	Acd	Acd	Alk		Alk*	Alk		Acd	Acd		Acd				Acd	Acd
	Gas	–	–		–	–		–	–		–				–	
B. xerosis	Acd	Acd	Alk		Acd	Alk		Acd	Acd		Acd				Alk	Acd
	Gas	–	–		–	–		–	–		–				–	
B. Hoffmanii	Acd	Alk	Alk		Alk	Alk		Alk	Alk		Alk				Alk	Alk
	Gas	–	–		–	–		–	–		–				–	
Diplococcus pneumoniæ	Acd	Acd	Acd		Acd	0	Acd							Acd		
	Gas		–		–	–								–		

Diplococ. intra-cell. mening.	Acd	Acd	0		0			0	0		Acd			0		
	Gas	—			—			—	—		—			—		
Gonococcus	Acd	Acd	0		0			0	0		0					
	Gas	—	—		—			—	—		—					
M. catarrhalis	Acd	0	0		0			0	0		0					
	Gas		—													
M. pharyngis siccus	Acd	Acd	0		Acd			Acd	0		Acd					
	Gas	—	—		—			—	—		—					
Diplococcus crassus	Acd	Acd	Acd		Acd			Acd	Acd		Acd					
	Gas	—	—		—			—	—		—					
Strept. pyogenes		Acd	Acd		Acd						Acd					Acd
		—	—		—						—					—
Strept. viridans		Acd			Acd											
		—			—											
Staph. pyogenes aureus		Acd	Acd		Acd											Acd
		—	—		—											—
M. tetragenus		Acd														
		—														

S = slight.
* = some strains.
0 = No acid but not known whether no change or alkaline.

CHARACTERS OF B. COLI – B. TYPHOSUS GROUPS

Names of Organisms.	Dextrose.	$H_2 : CO_2$	Lactose.	Saccharose.	Dulcit.	Adonit.	Liver broth.	Inulin.	Indol.	Nitrites.	Litmus milk.	Neutral red broth.	Gelatin liquefaction.	Esculin bile.	Endo medium	Conradi-Drigalski's medium.	Vosges-Proskauer's reaction.	Gram's stain.	Motility.	Morphology.	Litmus lactose agar.	NaOH red reaction
B. coli communis (Escherich).	+	$\frac{1+}{1}$	+	−	+	−	+	−	+	+	+	+	−	4 dys. +	+	red	−	−	slow +	rod	+	−
B. coli communior (Emmerich)	+	$\frac{1+}{1}$	+	+	+	−	+	−	±	+	+	+	−	−	+	red	−	...	−	rod	+	+
B. aerogenes 1..............	+	....	+	+	−	...	+	−	+	+	+	+	−	3 dys. +	+	red	.....	...	−	rod	+	−
B. aërogenes 2..............	+	....	+	+	+	...	+	−	+	+	+	+	−	8 dys. +	+	red	.....	...	+	rod	+	−
B. acidi lactici (Hueppe).....	+	$\frac{1+}{1}$	+	−	−	+	+	−	+	+	+	+	−	−	+	red	−	...	−	rod	+	−
B. lactis aerogenes (Escherich).	+	$\frac{1}{1+}$	+	+	−	+	+	−	±	+	+	+	−	1 dy. +	+	red	+	...	−	rod	+	+
Bact. aërogenes (Pfeiffer).....	+	$\frac{1}{1+}$	+	+	−	+	+	−	−	+	+	+	−	−	+	red	+	...	.....	rod	+	+
B. cloacæ (Jordan)..........	+	$\frac{1}{1+}$	+	+	−	−	+	−	−	+	+	+	+	5 dys. +	+	red	+	...	+	rod	+	+
B. choleræ suis.............	+	$\frac{1+}{1}$	−	−	−	...	+	...	−	+	alk	red	−	.....	−	blue	−	...	+	rod	−	
B. paratyphosus A..........	+	$\frac{1+}{1}$	−	−	+	...	+	...	−	...	+	....	−	.....	−	blue	−	...	+	rod	−	
B. paratyphosus B..........	+	$\frac{1+}{1}$	−	−	+	...	+	...	−	...	alk	...	−	.....	−	blue	−	...	+	rod	−	
B. typhosus................	−	....	−	−	−	−	−	−	−	+	−	−	−	−	−	blue	−	−	+	rod	−	

+ = positive; − = negative. ± = varieties of one species variable.

COMMON DISINFECTANTS

Mercuric chloride: ($HgCl_2$) White crystals.

Synonyms: mercury bichloride, corrosive sublimate, bichloride of mercury.

The stock solution (40% $HgCl_2$ in HCl) is prepared by mixing 1 part mercuric chloride with 2.5 parts commercial hydrochloric acid. This dissolves readily and aqueous solutions of any desired dilution may be made from it much more quickly than by the use of the salt.

The pure salt is soluble in 16 parts of cold water and 3 parts of boiling water.

Mercuric chloride 1 : 1000, the solution commonly used in the laboratory for disinfecting purposes, is prepared by adding to 2.5 c.c. of the stock solution, sufficient distilled water to make 1000 c.c. of solution.

As a germicide, mercuric chloride acts in solution by combining chemically with the protein of the microorganisms. Therefore its efficiency varies in inverse proportion to the amount of dead organic matter present.

Mercuric chloride is exceedingly corrosive as is also the acid in which it is originally dissolved; therefore it should not be placed in metal containers or agateware pails, cups, etc., if the enamel is chipped sufficiently to expose the metal.

Remember that mercuric chloride is a DEADLY POISON! Great care must be exercised in properly labelling all bottles, etc., containing it.

Phenol: (C_6H_5OH) long colorless crystals that become pink upon exposure to light and air.

Synonyms: *Carbolic acid,* phenic or phenylic acid, phenyl hydrate, hydroxybenzene (or -ol).

The stock solution (95% phenol) is prepared by adding 1 part of water to 19 parts (by weight) of crystalline phenol. Solution may be hastened by placing the vessel containing the crystals in a dish of warm water.

Note. When making up the stock solution or dilutions from the stock solution always have a bottle of ethyl alcohol at hand as a remedy for burns caused by phenol. 5% phenol is prepared by adding one part of 95% phenol to nineteen parts of distilled water.

Its value as a disinfectant is increased by the fact that it acts in the presence of albuminous substances. It does not corrode metals or destroy fabrics in a 5% solution.

Liquor cresolis compositus, U. S. P.

Cresol	500 gms.
Linseed oil	350 gms.
Potassium hydroxide	80 gms.
Water, a sufficient quantity to make	1000 gms.

Dissolve the potassium hydroxide in 50 gms. of water in a tared dish, add the linseed oil, and mix thoroughly. Then add the cresol and stir until a clear solution is produced. Finally add sufficient water to make the finished product weigh 1000 gms., or more briefly: mix equal parts by weight of cresol and linseed oil-potash soap (*Sapo mollis*, U. S. P.).

This mixture is a thick, dark, amber-colored fluid which mixes readily with water in all proportions to form a clear, soapy solution. A 3% or 4% solution will accomplish the same results as 5% phenol. It is not interfered with by albuminous substances and does not destroy metals or fabrics.

Tincture of iodin, U. S. P.

Iodin	70 gms.
Potassium iodid	50 gms.
Alcohol, sufficient to make	1000 c.c.

Triturate the iodin and potassium iodid in a mortar to a coarse powder and transfer at once to a graduated flask. Rinse the mortar with several successive portions

of alcohol and pour the rinsings in the bottle; then add alcohol, shaking occasionally until the iodin and potassium iodid are all dissolved and the finished tincture measures 1000 c.c.

SOLUTIONS FOR CLEANING GLASSWARE

Chromic acid cleaning solution for cleaning glassware:

Potassium or sodium dichromate	60 gms.
Commercial sulphuric acid	60 c.c.
Water	1000 c.c.

Prepare in a flask resistant to heat, never in a heavy glass jar.

Add the potassium dichromate to about 500 c.c. water; shake well and add the sulphuric acid gradually, continually shaking with a rotary motion. The remaining water may then be added. The potassium dichromate should be all dissolved before using the solution.

This solution may be used repeatedly until oxidized to a dark green color. Heat will hasten its action.

Chromic acid cleaning solution is especially valuable for removing traces of oxidizable organic matter and neutralizing any free alkali adhering to glassware. However, effort should be made to previously remove as much extraneous matter as possible with water and a suitable brush before treating with this solution. This will economize time.

Caution. This solution contains sufficient sulphuric acid to destroy fabrics, bristles of brushes, and corrodes metal quickly. For this reason neither cloth nor brushes should be used as an immediate aid to this cleaning agent, *nor should this solution be placed in agateware utensils if the enamel is chipped, exposing the metal.*

If this solution is used cold, leave the glassware containing it, over night *on top* of desk, *never* inside of desk.

Sodium hydroxide solution for cleaning glassware and absorbing CO_2:

Sodium hydroxide, sticks	100 gms.
Water	1000 c.c.

Use only once if the glassware is very dirty.

This solution is invaluable for cleaning greasy flasks, pipettes, etc.

Caution. This solution should not be left in contact with any glassware longer than thirty minutes as it etches the glass.

A sodium hydroxide solution of this strength is very corrosive, attacking cloth, laboratory desk tops, etc., and, therefore, should be wiped up immediately if spilled.

This strength may also be employed to absorb CO_2 in fermentation tube cultures of gas-producing organisms.

STANDARD SOLUTIONS

A. Preparation of N/10 Na_2CO_3 from titration against which normal acid is prepared.

1. Dry finely powdered chemically pure Na_2CO_3 in a drying oven at 105° C. for two hours.

2. Weigh out carefully and as accurately as possible 5.3 gms. of the dried salt.

3. Dissolve in distilled water which has been boiled previously to expel CO_2 and then cooled.

4. Make up solution to one liter, using a calibrated volumetric flask and observing temperature for which the flask was calibrated.

5. Keep this N/10 solution of Na_2CO_3 in a stoppered bottle. It should be used as soon as possible after preparation, as the Na_2CO_3 acts upon the glass and thus deteriorates.

B. Preparation of N/1, N/10 and N/20 HCl.

1. Measure out 77.5 c.c. HCl (sp.gr. 1.20) or 138 c.c.

HCl (sp.gr. 1.12) and make up to one liter with distilled water. This makes a solution just a little stronger than normal.

2. To determine its exact strength, titrate 5 c.c. with N/10 Na_2CO_3, using phenolphthalein as the indicator.

3. Run check determinations, which should check within one- or two-tenths of a cubic centimeter.

4. From results, calculate by proportion how much a liter of the solution should be diluted to make it N/1. e.g.:

5 c.c. HCl was neutralized by 55 c.c. N/10 Na_2CO_3

$$\therefore \quad \text{HCl is N/1.1}$$

By proportion:

$$N/1 : N/1.1 :: 1000 : x$$

$$x = 1100$$

Hence each liter of the HCl solution should be diluted to 1100 c.c. with distilled water to make a N/1 solution of HCl.

5. N/10 and N/20 solutions of HCl can be made by making the proper dilutions. *Always use calibrated flasks and burettes when making these dilutions.*

C. Preparation of N/1 and N/20 NaOH.

1. Weigh out roughly 41 gms. of chemically pure NaOH.

2. Dissolve in distilled water, which has been boiled to expel CO_2 and then cooled.

3. Make up to one liter, using a calibrated volumetric flask and observing the temperature for which it was calibrated. This makes a solution a little stronger than normal.

4. Determine its exact strength by titration with N/10 HCl.

5. Proceed from this point as in the preparation of N/1 HCl.

6. N/10 and N/20 solutions can be made from the N/1 solution as in the preparation of N/10 and N/20 HCl.

INDICATORS

Phenolphthalein, indicator for titration:

Phenolphthalein	0.5 gm.
50% alcohol (neutral)	100.0 c.c.

A drop of a weak solution of alkali should produce permanent pink color when added to a small amount of this solution. Phenolphthalein is colorless in the presence of acid.

Kahlbaum's azolitmin solution: Dissolve 2.5 gms. of Kahlbaum's azolitmin in 100 c.c. distilled water by heating in steam for half an hour. Filter (this will filter much more readily if allowed to settle for some time; decant upon the filter). Sterilize by heating fifteen minutes each day on three successive days. Sterilization is necessary, otherwise molds and other microorganisms will grow on the organic material present, often changing the reaction.

A solution of litmus or azolitmin is often added to sugar and other media before sterilization for the purpose of detecting microorganisms which produce a change in the reaction of the media.

Litmus is a mixture of dyes obtained from the lichens *Roccella* and *Lecanora* by allowing them to ferment after the addition of ammonia and potassium carbonate. When the mass has assumed a deep blue color, the liquid is pressed out, absorbed by chalk or gypsum, and dried.

Merck's purified litmus, often used in bacteriological work, is made from commercial litmus solution by freeing it from the red pigment orcin, and drying without absorbing it by means of chalk or gypsum.

Azolitmin is a purified pigment from litmus.

SALT SOLUTIONS

Physiological salt solutions for immunity work, dilution flasks, etc.:

Sodium chloride, c.p.	8.5 gms.
Distilled water	1000.0 c.c.

Chemically pure sodium chloride must be used for immunity work, especially for animal injection. For dilution flasks the best grade of cooking salt serves the purpose. *Salt prepared for table use cannot be used on account of its starch content.*

Normal salt solution for dilution purposes, etc., *not for immunity work:*

Sodium chloride, best commercial grade	60 gms.
Distilled water	1000 c.c.

Citrated salt solution for used in demonstrating opsonins:

Sodium chloride, c.p.	8.5 gms.
Sodium citrate	15.0 gms.
Distilled water	1000.0 c.c.

TEST SOLUTIONS

Ehrlich's test solution for indol production:
Solution I.

Para-dimethyl-amido-benzaldehyde	4 gms.
96% alcohol	380 c.c.
HCl, conc.	80 c.c.

Solution II. Saturated watery solution of potassium persulphate (oxidizing agent).

See Exercise 44, Part I, for the method of the test.

Nitrate test solutions:

I. Phenolsulphonic acid.

1. Mix 3 gms. of pure crystallized phenol with 37 gms. of c.p. concentrated sulphuric acid (20.1 c.c., sp.gr. 1.84) in a round-bottom flask.

2. Heat for six hours in a water bath at 100° C., keeping the flask submerged the whole time.

This may crystallize on cooling, but it can be brought into solution easily by heat.

Directions for making this test will be noted in Exercise 45, Part I.

II. Diphenylamin. A solution of 2% diphenylamin in sulphuric acid when added to a liquid containing nitrates *or nitrites* gives a blue color.

Diphenylamin	2 gms.
Sulphuric acid, c.p. conc.	100 c.c.

Nitrite test solutions:

Solution I. 8.0 gms. sulphanilic acid dissolved in 1000 c.c. of 5N acetic acid (sp.gr. 1.041).

Solution II. 5.0 gms. *a*-naphthylamin dissolved in 1000 c.c. of 5N acetic acid. These solutions should be kept separate and mixed in equal parts just before use.

Nessler's solution, for free ammonia:

1. Dissolve 62.5 gms. of potassium iodid in 250 c.c. of distilled water. Reserve about 10 c.c. of this solution.

2. Add gradually to the main portion a cold saturated solution of mercuric chloride, stirring constantly and increasing the quantity of mercuric chloride until a bright, permanent precipitate is formed.

3. Now add the reserved potassium iodid solution and again add the saturated mercuric chloride solution, cautiously and with constant stirring until a distinct though slight red precipitate remains.

4. Dissolve 150 gms. of caustic potash in 150 c.c. dis-

tilled water, allow the solution to cool and add it to the above solution.

5. Dilute to one liter with distilled water.
6. Allow to stand for one week and decant for use.

MOUNTING MEDIA

Canada balsam for making permanent mounts of microscopic preparations:

Canada balsam, dry, hard, for microscopic use	4 parts
Xylol	3 parts

This gives a mounting medium of about the right consistency. It should not "thread" when a drop is taken out with the glass rod. Balsam should be kept in a bottle stoppered with a glass bell-stopper, and having a rim arranged so that the excess of balsam taken upon the glass rod can be drained off.

Immersion oil for oil immersion objectives.

It is necessary that the immersion oil have practically the same index of refraction as glass in order to avoid dispersion of any of the light rays. Cedar wood oil having a refractive index of 1.515 to 1.520 is the usual medium interposed between the specimen and the oil immersion objective as it has approximately the same index of refraction as crown glass, 1.518. The refractive index of air is 1.000.

Chinese ink:

Burri's "Pelikan" Chinese ink	1 part.
Distilled water	7 parts.

Tube, using 8 to 10 c.c. per tube, sterilize in the autoclav and allow to stand two or three weeks without disturbing, for sedimentation to take place. It is to be used without shaking or disturbing any more than necessary.

STAINS

Methylen blue for differentiating living from dead yeast cells:

Methylen blue	0.1 gm.
Distilled water	1000.0 c.c.

Aqueous-alcoholic stains, fuchsin, methylen blue and gentian violet:

1. A saturated alcoholic solution of a stain is prepared by shaking frequently about 10 gms. of the stain with 100 c.c. of absolute alcohol. If the stain dissolves quickly, add more dry stain. The alcoholic solution should be slightly supersaturated.

2. Allow the undissolved stain to settle over night.

3. Decant.

4. Dilute 1 part of the alcoholic solution with 9 parts of distilled water.

Note 1. If 95% alcohol is used instead of absolute alcohol to dissolve the stain, the dilution should be made 1 : 7.

Note 2. These aqueous solutions may not keep longer than about a month, while the saturated alcoholic solutions keep indefinitely.

Note 3. The vegetative forms of bacteria stain more or less readily with all aqueous-alcoholic stains but *not with saturated alcoholic stains.* Acid-fast bacteria, e.g., *Bact. tuberculosis,* are the exception to the former.

Anilin-water gentian violet:

1. Shake 5 c.c. of anilin oil vigorously with 100 c.c. of distilled water in a stoppered bottle for several minutes.

2. Filter through a wet filter immediately before use.

3. Add 1 part of saturated alcoholic solution of gentian violet to 9 parts of the freshly prepared anilin-water and filter immediately before use.

Note. Anilin-water stains do not keep longer than about a week. The stock solutions will keep indefinitely if kept separate.

Ziehl-Nielson's carbol-fuchsin.

Solution A.

Basic fuchsin	1 gm.
Absolute alcohol	10 c.c.

Solution B.

Carbolic acid	5 gms.
Distilled water	100 c.c.

1. Dissolve the fuchsin in the absolute alcohol. (*Solution A.*)
2. Dissolve the carbolic acid in the distilled water (*Solution B*).

Note. *Solutions A and B* will keep indefinitely if kept separate.

3. Mix in the proportion of 10 c.c. of *solution A* to 100 c.c. of *solution B.*

Note. If *A* and *B* do not mix readily, warm slightly and add a few drops of absolute alcohol.

4. Filter.

Loeffler's alkaline methylen blue.

Saturated alcoholic solution of methylen blue	30 c.c.
Potassium hydrate, 0.1% aqueous solution	100 c.c.

Stabilized Gentian Violet. (Stovall and Nicholls, 1916.) To prevent the usual deterioration of gentian violet, the following stain is proposed:

Aniline	28 c.c.
Gentian violet	8 gms.
95 per cent alcohol	100 c.c.
Normal hydrochloric acid	5 c.c.
Distilled water	1000 c.c.

The gentian violet should be dissolved in the alcohol. The aniline and hydrochloric acid may be mixed and diluted to

900 c.c. Filter these solutions and mix with a subsequent filtration.

SOLUTIONS FOR USE IN STAINING

Aceton-alcohol for decolorizing in Gram's method of staining:

Aceton	10 c.c.
Absolute alcohol	100 c.c.

Acetic acid-alcohol for clearing in making impression preparations (also used for decolorizing in ordinary method of spore-staining):

Alcohol, 90%	2 parts
Acetic acid, 1%	1 part.

Mordant for staining flagella:

Tannin, 20%	10 c.c.
Ferrous sulphate, cold saturated solution	8 c.c.
Fuchsin, cold saturated solution in absolute alcohol	1 c.c.

Lugol's iodin solution, for use in Gram's staining method:

Iodin	1 gm.
Potassium iodid	3 gms.
Distilled water	300 c.c.

NOTES ON HYDROGEN-ION CONCENTRATION

Brief discussion of theory. The phenomena that come into play in dealing with dilute aqueous solutions of acids, bases or salts are those associated with the *ions* of these substances with very few exceptions. Thus, a salt when dissolved in pure water is partly dissociated into particles called ions which carry positive or negative electrical charges, as,

$$\underset{\text{undissociated molecule}}{MX} \rightleftarrows \underset{\text{cation}}{M^+} + \underset{\text{anion}}{X^-}$$

(M representing the basic constituent, as potassium (K), sodium (Na) and X the acidic constituent, as chlorine (Cl), the sulphate radical, (SO_4) etc. of the salt molecule. The electrolytic dissociation among other factors depends upon the nature of the salt and upon its concentration in the solution. The variation in degree of ionization of various normal acids, bases and salts at 18° C. is shown in the following table:

Normal Acid.	Per cent Ionized.	Normal Base	Per cent Ionized.	Normal Salts.	Per cent Ionized.
Nitric	82.00	Potassium hydroxide	77.00	Potassium Chloride	74.00
Perchloric	88.00	Sodium hydroxide	73.00	Ammonnium Chloride	75.00
Sulphuric	51.00	Barium hydroxide	92.00	Potassium Nitrate	64.00
Carbonic	00.17	Ammonium hydroxide	00.50		
Hydrosulphuric	00.07				

The more dilute the solution, the greater the extent of electrolytic dissociation. Likewise acids that are largely dissociated in aqueous solutions, as hydrochloric, nitric, and sulphuric, are termed strong acids; whereas those that are only feebly ionized, as acetic, boric, and carbonic, are

termed weak acids. Similarly there are termed strong bases as sodium, potassium, and barium hydroxides and weak bases as ammonium hydroxide.

The degree of ionization must not be confused with the absolute concentration of the ions. In order to compute the latter, both the degree of ionization and the concentration of the solution must be known. The variation in degree of electrolytic dissociation for the case of hydrochloric acid is shown in the table below.

HYDROCHLORIC ACID

Concentration.		Degree of Dissociation.
1.	Normal	79.2
0.1	"	92.4
0.01	"	97.1
0.001	"	98.8
0.0001	"	99.5

Hydrochloric acid dissociates in the following manner in aqueous solution:

$$HCl \rightleftarrows H^{+} + Cl^{-}$$

The ($\rightleftarrows$) sign indicates that the process is reversible. The more dilute the solution, the more completely ionized the acid becomes. The concentration of the hydrogen ion in a normal solution (in which the degree of ionization is 79.2%) is:

$$\text{Concentration } (H^{+}) = 1.0 \times 0.792 = 0.792 \text{ N.}$$

Since, for every hydrogen ion there is one chlorine ion, the concentration of the latter will be 0.792 N also, or:

$$\text{Concentration } (Cl^{-}) = 1.0 \times 0.792 = 0.792 \text{ N.}$$

This means that the solution is 0.792 normal with respect to the hydrogen ion.

In case of the more dilute acid, say 0.0001 N, the hydrogen ion concentration will be:

$$C(H^+) = 0.0001 \times 0.995 = 0.0000995 \text{ N.}$$

or the number of grams of hydrogen will be:

$$0.0000995 \times 1.008 = 0.0001002960 \text{ gram.}$$

Thus, it will be seen that although the degree of electrolytic dissociation is much greater in the case of the more dilute acid, the absolute ion concentration is much less than that of the normal solution.

The method of expressing hydrogen ion concentrations. In bacteriological work the solutions used have a hydrogen ion concentration which is very much less than those ordinarily used in chemical work. However, the standard remains the same, viz., the normal solution, which contains the gram equivalent of an acid or base in one liter. Since these values are so much less and contain a larger number of figures, they are more conveniently written in an abbreviated form e.g.: 0.0001 and 0.000,0001 are written 1×10^{-4} and 1×10^{-7} respectively.

Experiment has shown that even pure water dissociates into ions as follows:

$$H_2O \rightleftarrows H^+ + OH^-.$$

Experiment has also shown, that for a given temperature, the product of the two ion concentrations is a perfectly definite constant and at 25° C. is equal to 1×10^{-14}, or

$$C(_{H^+}) \times C(OH^-) = 1 \times 10^{-14}.$$

In pure water the number of hydrogen ions is equal to the number of hydroxyl ions. Therefore, the hydrogen ion concentration of pure water is:

$$C(_{H^+}) = \sqrt{1 \times 10^{-14}} = 1 \times 10^{-7}.$$

This concentration of hydrogen or hydroxyl ions (1×10^{-7}) is called the *true neutral point*. A solution having a hydrogen ion concentration greater than this, e.g., 1×10^{-4} is acid; while a solution with a hydrogen ion concentration less than 1×10^{-7}, as 1×10^{-9} is alkaline.

Knowing the hydrogen ion concentration of pure water; the hydrogen ion concentration of a dilute solution of alkali (which, although smaller than that of water, is by no means equal to zero) may be computed Thus, assuming 0.0001 N sodium hydroxide to be completely dissociated electrically, the hydroxyl concentration is:

$$C(OH^-) = 0.0001\times1.0 = 1\times10^{-4}$$

The hydrogen ion concentration of such a solution may be found by substituting the value of the hydroxyl ion concentration in the equation:

$$C_{H^+}\times C_{OH^-} = 1\times10^{-14}$$

Whence,

$$C_{H^+}\times1\times10^{-4} = 1\times10^{-14}$$

And

$$C_{H^+} = \frac{1\times10^{-14}}{1\times10^{-4}} = 1\times10^{-10}.$$

Thus, it will be seen that the hydrogen ion concentration of such a solution is very small and since it is less than that of pure water (1×10^{-7}), the solution would be considered alkaline.

Since even the abbreviated form of such a number as 0.01 viz., 1×10^{-2} is rather inconvenient to write and also for convenience in plotting curves showing variations in hydrogen ion concentration under various conditions, a value, which is the logarithm of the reciprocal of the hydrogen ion concentration, is frequently used. To this value Sørensen gave the symbol P_H^+ which is usually written P_H. Another reason (Clark and Lubs) is that when the electrometric method is

used for determining hydrogen ion concentration the potentials measured are introduced into an equation which contains the term $\log \frac{1}{H}$. "The value of this term is obtained very directly and serves to characterize a hydrogen ion concentration quite as well as if the calculation were carried further to obtain the actual value of (H)."

Its relation to the hydrogen ion concentration may be indicated as follows:

By definition, $$P_H = \log \frac{1}{C_{H^+}},$$

wherefore, if $C_{H^+} = 1 \times 10^{-2}$,

$$P_H = \log \frac{1}{1 \times 10^{-2}} = \log 100.$$

The logarithm of 100 is 2, whence

$$P_H = 2.$$

Similarly, where $C_H = 1.2 \times 10^{-6}$,

$$P_H = \log \frac{1}{1.2 \times 10^{-6}} = \log \frac{1}{0.0000012} = 5.92.$$

Indicators. An indicator is a weak organic base or acid which changes color within certain specific ranges of hydrogen ion concentration. When a solution of a base is titrated with a standard acid, or vice versa, the end point of the titration should occur when the amounts of acid and of base employed are exactly equivalent to each other. The composition of the solution at the end of the titration should be equal to what would be obtained by dissolving the pure salt in the proper amount of pure water. The "end point" of the indicator frequently used in volumetric analysis, while sufficiently accurate for analytical purposes, is rarely that of exact neutrality. Owing to the germicidal action of

hydrogen ions and to the fact that certain bacteria grow only within well defined zones of hydrogen ion concentration, the neutral point obtained with the ordinary titration indicators is wholly inadequate in adjusting bacterial media.

The weakness of the indicator is essential, since the weaker it is the more its dissociation will depend upon the variation in hydrogen or hydroxyl ion concentration of the solution during the process of titration and hence the sharper its end point will be. This is apparent in the following cases. Phenolphthalein, commonly used as an indicator, is one of the weakest acids known and, as a consequence, is only feebly ionized according to the scheme:

$$\underset{\text{Undissociated molecule (colorless)}}{HP} \rightleftarrows H^+ + \underset{\text{Red colored}}{P^-}.$$

When a base is added, however, a salt is formed between the base and indicator and, as salts are more highly dissociated than the weak acid or base from which they are formed, the concentration of the phenolphthalein ion increases and, consequently, the solution becomes red.

In the case of methyl orange we have a weak basic indicator. The undissociated molecule is yellow, the cation red:

$$\underset{\text{Undissociated molecule (yellow)}}{BOH} \rightleftarrows \underset{\text{(red)}}{B^+} + \underset{\text{(colorless)}}{OH^-}$$

Methyl orange is a stronger base than phenolphthalein is an acid. With methyl orange in solution, we have sufficient B and BOH ions to cause color (combined). On adding hydrochloric acid (a strong acid) to methyl orange, we add hydrogen ions, and the reaction goes from left to right, and we have the hydrochloride of the indicator formed. It is highly dissociated, and we get the color of the B ions (red).

The mechanism of titration may be looked at from the standpoint of the strength of the two acids concerned. Starting with an acid solution containing phenolphthalein, we

add alkali drop by drop and the alkali distributes itself between the two acids. Herein is the advantage of having the indicator very weak—it gets only a small fraction of the base and this effect is accentuated by the fact that the ionization of the indicator is extremely slight in acid solution. When all the acid has been used up and sufficient alkali has been added the color begins to appear.

That, however, a condition of true neutrality is not reached at the so-called "end-point" of the titration is shown by the accompanying curve (Fig. 1) which represents the

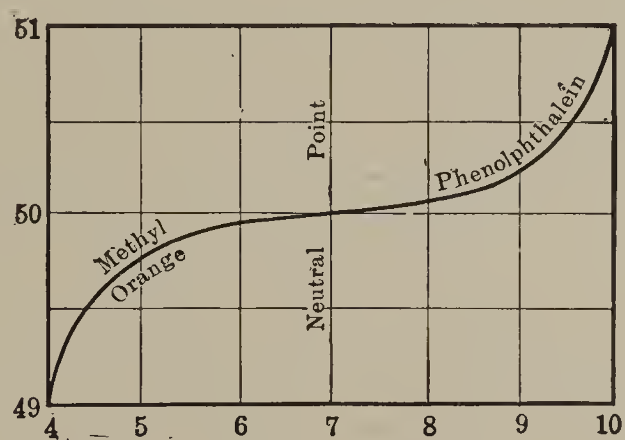


Fig 1.

change in hydrogen ion concentration when the titration of 50 c.c. of N/100 hydrochloric acid by N/100 sodium hydroxide is almost complete. The number of c.c. of alkali added are represented as ordinates, while the abscissæ represent the P_H values.

The two indicators are written in on the curve at the points at which their color change or "end points" occur. It will be noticed that with methyl orange as indicator the color change occurs at a P_H value of from 4 to 5, namely, when about 49.8 c.c. of alkali have been added, whereas, with phenolphthalein the pink colored end point is reached when 50.06 c.c. of alkali have been added. The P_H value at this end point is 9. By making careful check runs the true neutral point may be more closely approached than this, but

in any case the true hydrogen ion concentration is somewhat in question.

Indicators Recommended by Clark and Lubs. In view of the great need of indicators that are far more brilliant and more sensitive to slight changes in acidity, Clark and Lubs after careful research have recommended the following list:

Chemical Name.	Common Name.		Color Change.	Range PH
Thymolsulfonephthalein (acid range)	Thymol blue	0.04	Red-yellow	1.2–2.8
Tetrabromophenolsulfonephthalein	Brom phenol blue	0.04	Yellow-blue	3.0–4.6
Orthocarboxybenzeneazodimethyl aniline	Methyl red	0.02	Red-yellow	4.4–6.0
Orthocarboxybenzeneazodipropylaniline	Propyl red	0.02	Red-yellow	4.8–6.4
Dibromoorthocresolsulfonephthalein	Brom creso purple	0.04	Yellow-purple	5.2–6.8
Dibromothymolsulfonephthalein	Brom thymol blue	0.04	Yellow-blue	6.0–7.6
Phenolsulfonephthalein	Phenol red	0.02	Yellow-red	6.8–8.4
Orthocresolsulfonephthalein	Cresol red	0.02	Yellow-red	7.2–8.8
Thymolsulfonephthalein (alkali range)	Thymol blue	0.04	Yellow-blue	8.0–9.6
Orthocresolphthalein	Cresol phthalein	0.02	Colorless-red	8.2–9.8

Preparation of Indicators for Use. The indicators may be secured in the powdered form. This has been found to be the most practical and satisfactory way to market the indicator. Concentrated or stock solutions may then be easily prepared from the powders.

Test solutions are made by diluting the stock solutions with 30 volumes of distilled water as needed.

Methyl red is prepared by dissolving 1 decigram of the

PREPARATION OF INDICATORS

Name	Grams	N/20 Na OH	Water to make.
Phenolsulphonephthalein	0.1	5.7 c.c.	15 c.c.
Cresolsulphonephthalein	0.1	5.3 c.c.	15 c.c.
* Thymolsulphonephthalein	0.1	4.3 c.c.	15 c.c.
* Tetrabromphenolsulphonephthalein	0.1	3.0 c.c.	15 c.c.
* Dibromocresolsulphonephthalein	0.1	3.7 c.c.	15 c.c.
* Dibromothymolsulphonephthalein	0.1	3.2 c.c.	15 c.c.

* Make in double strength.

powder in 300 c.c. of alcohol and diluting to 500 c.c. with distilled water.

Orthocresolphthalein is used in a 0.02% alcoholic solution.

In cases where alcohol or the acidity of the dye itself will not interfere with a test, alcoholic solutions of the acid dyes themselves may be used for the solutions. The concentrations of these alcoholic solutions may be approximately the same as those recommended for the aqueous sodium salts.

Standard solutions. It has been shown by a number of investigators that when acid or alkali are added to certain solutions that the solutions have the power to resist change in reaction. Fernbach and Huebert gave the name "tampon" to phosphate solutions exhibiting this property, later Sørensen used the term "puffer" and from this the English "buffer" came into use. This is illustrated in the following example (Clark and Lubs). If 1 c.c. of 0.01 N HCl is added to 1 liter of pure water ($P_H = 7.0$) the resulting solution would be about $P_H = 5.0$ and very toxic to bacteria. If, however, we added this same amount of acid to 1 liter of standard beef infusion having a $P_H = 7.0$, the resulting change in P_H would hardly be appreciable. The beef infusion we say is highly buffered. Buffer action then is defined as the ability of a solution to resist change in P_H through the addition or loss of acid or alkali.

The standard solutions used for this work are buffer solu-

tions with such well defined composition that they can be accurately reproduced and with P_H values accurately defined by hydrogen electrode measurements. Utmost care must be used in the preparation of the solution from very carefully purified substances and for very accurate work they should be checked electrometrically. The solutions are adjusted to the exact degree of hydrogen ion concentration by mixing varying quantities of a solution of pure sodium hydroxide (carbonate free), say, with a measured quantity of an acid salt, such as acid potassium phosphate, diluting to a given volume. In order to prepare standard solutions, the following special solutions are used: M/5 hydrochloric acid, M/5 sodium hydroxide, M/5 acid potassium phthalate, M/5 acid potassium phosphate, M/5 potassium chloride M/5 boric acid, and M/5 potassium chloride. The method of combining these so as to give solutions of standard hydrogen ion concentration is indicated in the following table after Clark and Lubs.

COMPOSITIONS OF MIXTURES GIVING P_H VALUES AT INTERVALS OF 0.2 AT 20° C.

KCl—HCl Mixtures

P_H	Composition.		
1.0	50 c.c. M/5 KCl	97.0 c.c. M/5 HCl	Dilute to 200 c.c.
1.2	50 c.c. M/5 KCl	64.5 c.c. M/5 HCl	Dilute to 200 c.c.
1.4	50 c.c. M/5 KCl	41.5 c.c. M/5 HCl	Dilute to 200 c.c.
1.6	50 c.c. M/5 KCl	26.3 c.c. M/5 HCl	Dilute to 200 c.c.
1.8	50 c.c. M/5 KCl	16.6 c.c. M/5 HCl	Dilute to 200 c.c.
2.0	50 c.c. M/5 KCl	10.6 c.c. M/5 HCl	Dilute to 200 c.c.
2.2	50 c.c. M/5 KCl	6.7 c.c. M/5 HCl	Dilute to 200 c.c.

Phthalate—HCl Mixtures

2.2	50 c.c. M/5 KH Phthalate	46.70 c.c. M/5 HCl	Dilute to 200 c.c.
2.4	50 c.c. M/5 KH Phthalate	39.60 c.c. M/5 HCl	Dilute to 200 c.c.
2.6	50 c.c. M/5 KH Phthalate	32.95 c.c. M/5 HCl	Dilute to 200 c.c.
2.8	50 c.c. M/5 KH Phthalate	26.42 c.c. M/5 HCl	Dilute to 200 c.c.
3.0	50 c.c. M/5 KH Phthalate	20.32 c.c. M/5 HCl	Dilute to 200 c.c.
3.2	50 c.c. M/5 KH Phthalate	14.70 c.c. M/5 HCl	Dilute to 200 c.c.
3.4	50 c.c. M/5 KH Phthalate	9.90 c.c. M/5 HCl	Dilute to 200 c.c.
3.6	50 c.c. M/5 KH Phthalate	5.97 c.c. M/5 HCl	Dilute to 200 c.c.
3.8	50 c.c. M/5 KH Phthalate	2.63 c.c. M/5 HCl	Dilute to 200 c.c.

Phthalate—NaOH Mixtures

P_H	Composition		
4.0	50 c.c. M/5 KH Phthalate	0.40 c.c. M/5 NaOH	Dilute to 200 c.c.
4.2	50 c.c. M/5 KH Phthalate	3.70 c.c. M/5 NaOH	Dilute to 200 c.c.
4.4	50 c.c. M/5 KH Phthalate	7.50 c.c. M/5 NaOH	Dilute to 200 c.c.
4.6	50 c.c. M/5 KH Phthalate	12.15 c.c. M/5 NaOH	Dilute to 200 c.c.
4.8	50 c.c. M/5 KH Phthalate	17.70 c.c. M/5 NaOH	Dilute to 200 c.c.
5.0	50 c.c. M/5 KH Phthalate	23.85 c.c. M/5 NaOH	Dilute to 200 c.c.
5.2	50 c.c. M/5 KH Phthalate	29.95 c.c. M/5 NaOH	Dilute to 200 c.c.
5.4	50 c.c. M/5 KH Phthalate	35.45 c.c. M/5 NaOH	Dilute to 200 c.c.
5.6	50 c.c. M/5 KH Phthalate	39.85 c.c. M/5 NaOH	Dilute to 200 c.c.
5.8	50 c.c. M/5 KH Phthalate	43.00 c.c. M/5 NaOH	Dilute to 200 c.c.
6.0	50 c.c. M/5 KH Phthalate	45.45 c.c. M/5 NaOH	Dilute to 200 c.c.
6.2	50 c.c. M/5 KH Phthalate	47.00 c.c. M/5 NaOH	Dilute to 200 c.c.

Phosphate—NaOH Mixtures

5.8	50 c.c. M/5 KH_2PO_4	3.72 c.c. M/5 NaOH	Dilute to 200 c.c.
6.0	50 c.c. M/5 KH_2PO_4	5.70 c.c. M/5 NaOH	Dilute to 200 c.c.
6.2	50 c.c. M/5 KH_2PO_4	8.60 c.c. M/5 NaOH	Dilute to 200 c.c.
6.4	50 c.c. M/5 KH_2PO_4	12.60 c.c. M/5 NaOH	Dilute to 200 c.c.
6.6	50 c.c. M/5 KH_2PO_4	17.80 c.c. M/5 NaOH	Dilute to 200 c.c.
6.8	50 c.c. M/5 KH_2PO_4	23.65 c.c. M/5 NaOH	Dilute to 200 c.c.
7.0	50 c.c. M/5 KH_2PO_4	29.63 c.c. M/5 NaOH	Dilute to 200 c.c.
7.2	50 c.c. M/5 KH_2PO_4	35.00 c.c. M/5 NaOH	Dilute to 200 c.c.
7.4	50 c.c. M/5 KH_2PO_4	39.50 c.c. M/5 NaOH	Dilute to 200 c.c.
7.6	50 c.c. M/5 KH_2PO_4	42.80 c.c. M/5 NaOH	Dilute to 200 c.c.
7.8	50 c.c. M/5 KH_2PO_4	45.20 c.c. M/5 NaOH	Dilute to 200 c.c.
8.0	50 c.c. M/5 KH_2PO_4	46.80 c.c. M/5 NaOH	Dilute to 200 c.c.

Boric Acid, KCl—NaOH Mixtures

7.8	50 c.c. M/5 H_3BO_3 M/5 KCl	2.61 c.c. M/5 NaOH	Dilute to 200 c.c.
8.0	50 c.c. M/5 H_3BO_3 M/5 KCl	3.97 c.c. M/5 NaOH	Dilute to 200 c.c.
8.2	50 c.c. M/5 H_3BO_3 M/5 KCl	5.90 c.c. M/5 NaOH	Dilute to 200 c.c.
8.4	50 c.c. M/5 H_3BO_3 M/5 KCl	8.50 c.c. M/5 NaOH	Dilute to 200 c.c.
8.6	50 c.c. M/5 H_3BO_3 M/5 KCl	12.00 c.c. M/5 NaOH	Dilute to 200 c.c.
8.8	50 c.c. M/5 H_3BO_3 M/5 KCl	16.30 c.c. M/5 NaOH	Dilute to 200 c.c.
9.0	50 c.c. M/5 H_3BO_3 M/5 KCl	21.30 c.c. M/5 NaOH	Dilute to 200 c.c.
9.2	50 c.c. M/5 H_3BO_3 M/5 KCl	26.70 c.c. M/5 NaOH	Dilute to 200 c.c.
9.4	50 c.c. M/5 H_3BO_3 M/5 KCl	32.00 c.c. M/5 NaOH	Dilute to 200 c.c.
9.6	50 c.c. M/5 H_3BO_3 M/5 KCl	36.85 c.c. M/5 NaOH	Dilute to 200 c.c.
9.8	50 c.c. M/5 H_3BO_3 M/5 KCl	40.80 c.c. M/5 NaOH	Dilute to 200 c.c.
10.0	50 c.c. M/5 H_3BO_3 M/5 KCl	43.90 c.c. M/5 NaOH	Dilute to 200 c.c.

The Preparation of "Conductivity" Water. Pure, redistilled, or so-called "conductivity" water is necessary for the crystallization of salts as a final step in their purification and in the preparation of the various standard solutions. This is best prepared in the following manner: Set up a Liebig condenser, the condensing tube of which is made of pure block tin tubing and bent so as to fit in the mouth of a 3 liter, constricted neck boiling flask. If a constricted neck flask is not obtainable, a 3 liter Pyrex flask provided with a stopper of pure block tin may be used equally well. To

three liters of distilled water in the flask add about 10 grams of barium hydroxide and a crystal or so of potassium permanganate—sufficient only to confer a pink tinge to the water. Heat to boiling and allow the whole apparatus to steam out for some time. A 2 liter bottle made of low solubility glass may be used as a receiver and should be thoroughly steamed out before use. The distillate should be collected in portions of about 100 c.c., the receiver being rinsed out with each successive portion which is rejected, until about one-third of the water has been distilled over. Only the middle third should be retained as "conductivity" water, the last fraction being discarded. Water thus prepared may be stored in opaque silica flasks or in bottles made of low solubility glass, the mouths of which are tightly closed by means of pure sheet tin-foil.

The Preparation of Stock Solutions (Clark and Lubs, Journal of Bacteriology, Vol. II, p. 20).

M/5 Potassium Chloride Solution. This solution will not be necessary except in the preparation of the most acid series of mixtures. The salt should be re-crystallized three or four times and dried in an oven at about 120° C. for two days. The fifth molecular solution contains 14.912 grams in 1 liter.

M/5 Acid Potassium Phthalate Solution. Acid potassium phthalate may be prepared by the method of Dodge (1915) modified as follows. Make up a concentrated potassium hydroxide solution by dissolving about 60 grams of a high grade sample in about 400 c.c. of water. To this add 50 grams of the commercial *resublimed* anhydrid of ortho phthalic acid. Test a cool portion of the solution with phenolphthalein. If the solution is still alkalin, add more phthalic anhydrid; if acid, add more KOH. When roughly adjusted to a slight pink with phenolphthalein (use a diluted portion for the final test; add as much more phthalic anhydrid as the solution contains and heat till all is dissolved. Filter while hot, and allow the crystallization to take place slowly. The crystals should be drained with suction and re-crystallized at least twice from distilled water. Dry the salt at 110–115° C. to constant weight. A fifth molecular solution contains 40.828 grams of the salt in 1 liter of solution.

M/5 Acid Potassium Phosphate Solution. A high grade commercial sample of the salt is re-crystallized at least three times from distilled

water and dried to constant weight at 110–115° C. A fifth molecular solution should contain in 1 liter 27.232 grams. The solution should be distinctly red with methyl red and distinctly blue with brom phenol blue.

M/5 Boric Acid M/5 Potassium Chloride. Boric acid should be re-crystallized several times from distilled water. It should be air dried (boric acid begins to lose "water of constitution" about 50° C.), in thin layers between filter paper and the constancy of weight established by drying small samples in a thin layer in a desiccator over $CaCl_2$. Purification of KCl has already been noted. It is added to the boric acid solution to bring the salt concentration in the borate mixtures to a point comparable with that of the phosphate mixtures so that colorimetric checks may be obtained with the two series where they overlap. One liter of the solution should contain 12.4048 grams of boric acid and 14.912 grams of potassium chlorid.

M/5 Sodium Hydroxid Solution. This solution is the most difficult to prepare, since it should be as free as possible from the carbonate. A solution of sufficient purity for the present purposes may be prepared from a high grade sample of the hydroxid in the following manner. Dissolve 100 grams NaOH in 100 c.c. of distilled water in a Jena or Pyrex glass Erlenmeyer flask. Cover the mouth of the flask with tin foil and allow the solution to stand over night till the carbonate has mostly settled. Then prepare a filter as follows. Cut a "hardened" filter paper to fit a Buchner funnel. Treat it with warm strong (1:1) NaOH solution. After a few minutes decant the sodium hydroxid and wash the paper first with absolute alcohol, then with dilute alcohol and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated but do not dry so that the paper curls. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod making sure that paper, under gentle suction, adheres well to the funnel, and draw the solution through with suction. The clear filtrate is now diluted quickly, after rough calculation, to a solution somewhat more concentrated than N/1. Withdraw 10 c.c. of this dilution and standardize roughly with an acid solution of known strength or with a sample of acid potassium phthalate. From this approximate standardization calculate the dilution required to furnish an M/5 solution. Make the required dilution with the least possible exposure, and pour the solution into a *paraffined* bottle to which a calibrated 50 c.c. burett and soda lime guard tubes have been attached. The solution should now be most carefully standardized. One of the simplest methods of doing this, and one which should be always used in this instance, is the method of Dodge (1915) in which use is made of the acid potassium phthalate purified as already described. Weigh out accu-

rately on a chemical balance with standardized weights several portions of the salt of about 1.6 grams each. Dissolve in 20 c.c. distilled water and add 4 drops phenolphthalein. Pass a stream of CO_2-free air through the solution and titrate with alkali till a faint but distinct and permanent pink is developed. It is preferable to use a factor with the solution rather then attempt adjustment to an exact M/5 solution.

M/5 Hydrochloric Acid Solution. Dilute a high grade of hydrochloric acid solution to about 20% and distill. Dilute the distillate to approximately M/5 and standardize with the sodium hydroxid solution previously described. If convenient, it is well to standardize this solution carefully by the silver chloride method and check with standardized alkali.

The only solution which it is absolutely necessary to protect from the CO_2 of the atmosphere is the sodium hydroxid solution. Therefore, all but this solution may be stored in ordinary bottles of resistant glass. The salt solutions, if adjusted to exactly M/5, may be measured from clean calibrated pipettes.

Method of procedure. Determination of the hydrogen ion concentration of media by the colorimetric method.

The determination of the hydrogen ion concentration (abbreviated as P_H) is one of the most important steps in the preparation of media. It has been clearly shown that the titrimetric method for the determination of true reaction is not satisfactory and is inaccurate when applied to culture media. It has also been shown that two media adjusted to the same " degree of titratable acidity " may have widely divergent hydrogen ion concentrations. Since it is the hydrogen ion concentration and not the " titratable reaction " which effects the growth of microorganisms, the former method is preferable.

Apparatus. Standard solutions; standard indicators (see appendix); comparator; uniform test tubes; 5 c.c. and 10 c.c. pipettes; culture medium to be tested.

1. Make up the medium, whose hydrogen ion concentration is to be determined, to a known volume, less than the final volume desired.

2. Prepare 10 c.c. of a standard solution of the P_H value desired in the medium and add the suitable indicator. (See page 412.)

3. Measure 10 c.c. of the medium whose P_H value is to be determined in a test tube. Add 10 drops of suitable indicator.

4. Compare with standard buffer solution by use of a comparator, using compensating blanks. Record results.

5. In case the medium does not have the desired reaction and you wish to adjust it proceed as follows:

6. Measure accurately 5 c.c. of the medium and dilute to 25 c.c. with distilled water.

7. Titrate the diluted medium with N/20 NaOH in the presence of the (suitable) indicator used above until the color corresponds with that of the freshly prepared standard using a comparator.

8. Compute and add the necessary amount of normal acid or alkali to bring the medium to the desired hydrogen ion concentration.

9. Check P_H values of the medium after the addition of each amount of normal acid or alkali, also after sterilizing, especially if a precipitate is formed.

Example. Desired P_H value of medium	= 7.2
Indicator covering that range	= Phenol red
Amount of Standard Solution * to use	= 10 c.c.
Burette reading after titrating	= 4.6
Burette reading before titrating	= 3.9
No. of c.c. N–20 NaOH to neutralize 5 c.c.	= 0.7 c.c.
No. of c.c. N–20 NaOH to neutralize 1000 c.c.	= 1000 : X : : 5 : 0.7 X = 140
No. of c.c. N–1 NaOH to add per liter of medium for neutralization	= 140 ÷ 20 or 7 c.c.

Suppose the P_H value is determined after the addition of N/1 NaOH and found to be 7.1 or 7.3. This would be near enough to the value desired for most purposes. For finer adjustments proceed as follows:

Finer adjustments. 1. Dilute 5 c.c. of the adjusted medium to 25 c.c. and add suitable indicator.

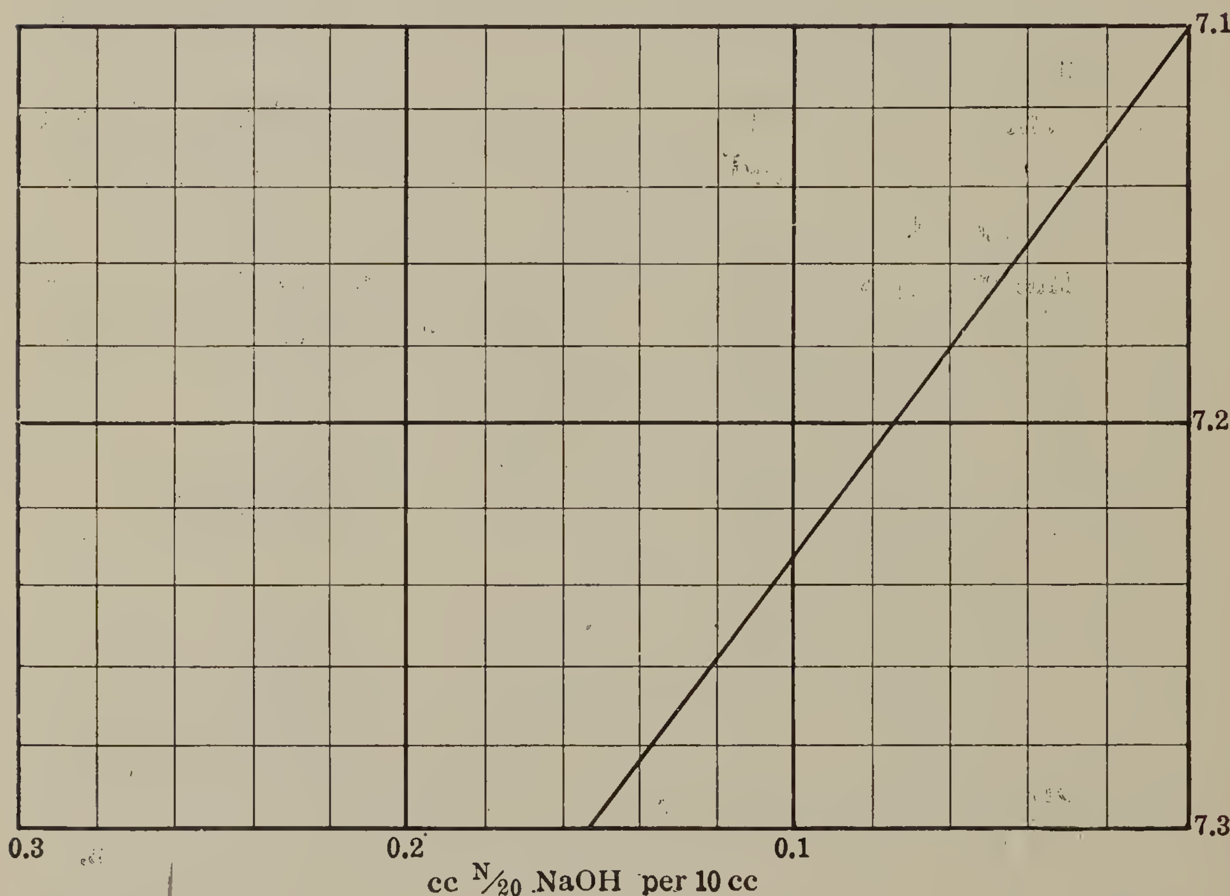
* 50 c.c. M/5 KH_2PO_4+35 c.c. M/5 NaOH and dilute to 200 c.c.; take 10 c.c.)

2. Compare with standard solution as before.

3. If it does not agree, titrate to the P_H value of (approximately) the next higher standard solution.

4. Plat these values on suitable coordinate paper and determine from graph the exact amount of normal acid or alkali to add to bring the desired P_H value.

Example. Original P_H value = 7.1
Amount of N/20 NaOH added to bring medium up to desired color = .16 c.c.
Final P_H value = 7.3
Desired P_H value = 7.2



Interpolating, it shows that 7.2 would have been reached on the addition of 0.077 c.c. N/20 NaOH to the 5 c.c. of medium. Hence to a liter of the medium, it would be necessary to add

5 c.c. : 0.077 = 1000 : X
X = 17.4 c.c. N/20 NaOH or 0.87 c.c. of N/1 NaOH

For accurate work, each determination should be checked up by the *electrometric method.*

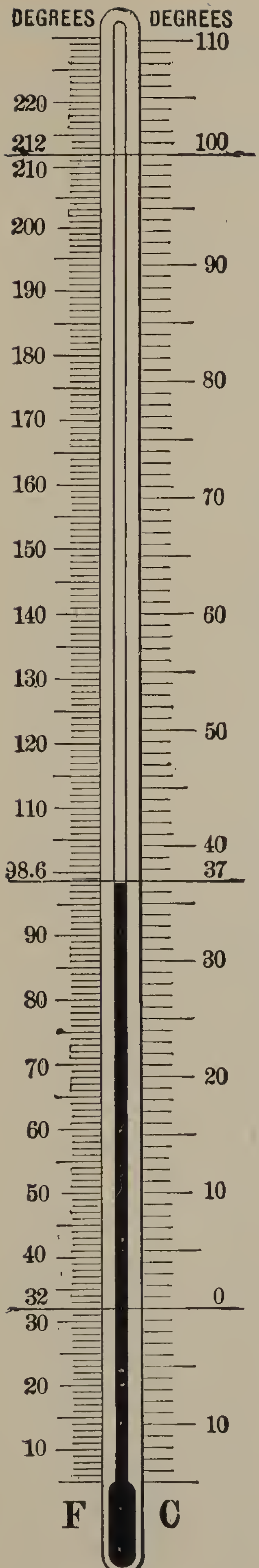


FIG. 74.—Comparison Fahrenheit-Centigrade Scale.

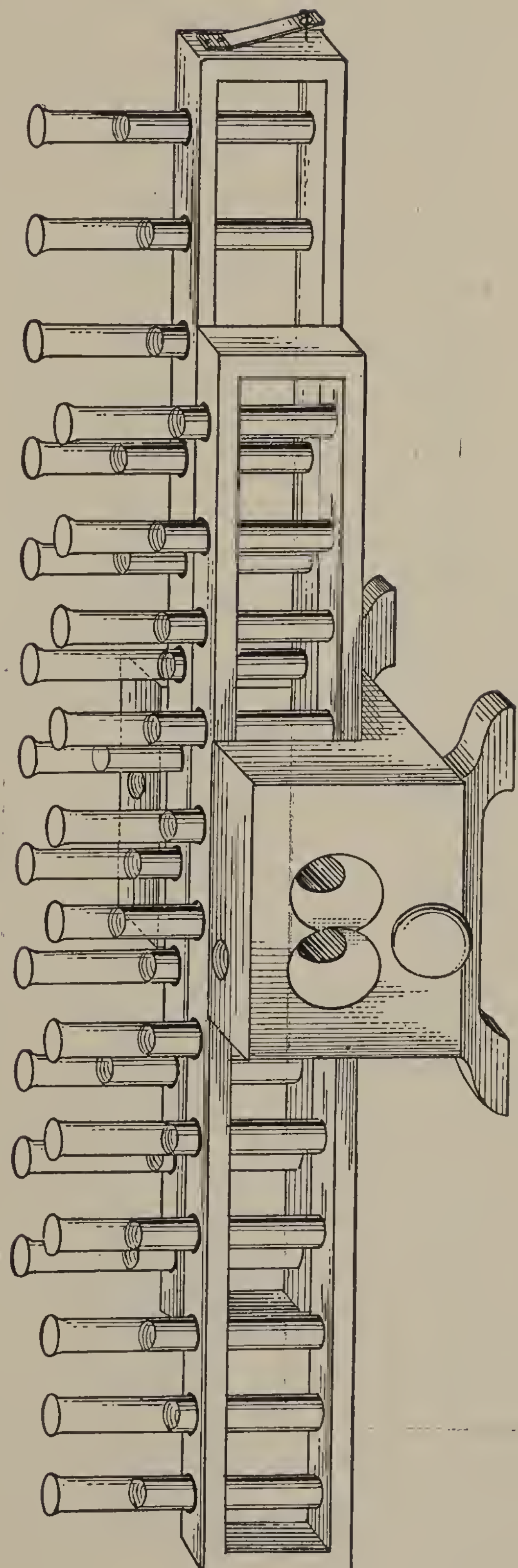


FIG. 75.—The Cooledge Comparator (Cen. Sci. Co.).

STEAM TEMPERATURE PRESSURE TABLE

Temperature Centigrade.	Mm. of Hg.	Pounds per sq.in. Absolute Pressure.	Atmospheres.
Degrees.			
98	707.1	13.7	0.93
99	733.1	14.2	0.96
100	760.0	14.7	1.00
101	787.8	15.2	1.03
102	816.0	15.8	1.07
103	845.2	16.3	1.11
104	875.4	16.9	1.15
105	906.4	17.5	1.19
106	938.3	18.1	1.23
107	971.1	18.8	1.27
108	1004.9	19.4	1.32
109	1039.6	20.1	1.36
110	1075.3	20.8	1.41
111	1112.0	21.5	1.46
112	1149.8	22.2	1.51
113	1188.6	22.9	1.56
114	1228.4	23.7	1.61
115	1269.4	24.5	1.67
116	1311.4	25.3	1.72
117	1354.6	26.2	1.78
118	1399.0	27.0	1.84
119	1444.5	27.9	1.90
120	1491.2	28.8	1.96
121	1539.2	29.7	2.02
122	1588.4	30.7	2.09
123	1638.9	31.7	2.15
124	1690.7	32.7	2.22
125	1743.8	33.7	2.29

FORMULAE FOR CONVERSION OF DEGREES OF TEMPERATURE ON ONE SCALE INTO DEGREES ON ANOTHER

Centigrade (Celsius) scale:	Freezing-point = 0°; boiling-point = 100°.
Fahrenheit scale:	Freezing-point = 32°; boiling-point = 212°.
Réaumur:	Freezing-point = 0°; boiling-point = 80°.

Degrees C $\times 1.8 + 32$ = Degrees F. Degrees $\frac{(F-32)4}{9}$ = Degrees R.

Degrees $\frac{F-32}{1.8}$ = Degrees C. Degrees $\frac{R \times 5}{4}$ = Degrees C.

Degrees $\frac{R \times 9}{4} + 32$ = Degrees F. Degrees $\frac{C \times 4}{5}$ = Degrees R.

ALCOHOL BY VOLUME

Tralles

(From the Chemiker Kalender, published by Julius Springer, Berlin.)

Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.	Per Cent by Vol.	Specific Gravity.
1	0.9976	26	0.9689	51	0.9315	76	0.8739
2	0.9961	27	0.9679	52	0.9295	77	0.8712
3	0.9947	28	0.9668	53	0.9255	78	0.8685
4	0.9933	29	0.9657	54	0.9254	79	0.8658
5	0.9919	30	0.9646	55	0.9234	80	0.8631
6	0.9906	31	0.9634	56	0.9213	81	0.8603
7	0.9893	32	0.9622	57	0.9192	82	0.8575
8	0.9881	33	0.9609	58	0.9170	83	0.8547
9	0.9869	34	0.9596	59	0.9148	84	0.8518
10	0.9857	35	0.9583	60	0.9126	85	0.8488
11	0.9845	36	0.9570	61	0.9104	86	0.8458
12	0.9834	37	0.9559	62	0.9082	87	0.8428
13	0.9823	38	0.9541	63	0.9059	88	0.8397
14	0.9812	39	0.9526	64	0.9036	89	0.8365
15	0.9802	40	0.9510	65	0.9013	90	0.8332
16	0.9791	41	0.9494	66	0.8989	91	0.8299
17	0.9781	42	0.9478	67	0.8965	92	0.8265
18	0.9771	43	0.9461	68	0.8941	93	0.8230
19	0.9761	44	0.9444	69	0.8917	94	0.8194
20	0.9751	45	0.9427	70	0.8892	95	0.8157
21	0.9741	46	0.9409	71	0.8867	96	0.8118
22	0.9731	47	0.9391	72	0.8842	97	0.8077
23	0.9720	48	0.9373	73	0.8817	98	0.8034
24	0.9710	49	0.9354	74	0.8791	99	0.7988
25	0.9700	50	0.9335	75	0.8765	100	0.7939

METRIC SYSTEM

Linear Measure

1000 millimicrons	= 1 micron (micromillimeter).
1000 microns	= 1 millimeter.
10 millimeters	= 1 centimeter.
10 centimeters	= 1 decimeter.
10 decimeters	= 1 meter.
10 meters	= 1 decameter.
10 decameters	= 1 hectometer.
10 hectometers	= 1 kilometer.
10 kilometers	= 1 myriameter.

The unit of length, one meter, is equal to $\frac{1}{1,000,000}$ part of the distance measured on a meridian of the earth from the equator to the pole and equals about 39.37 inches.

Square Measure

1,000,000 sq. millimicrons	= 1 sq. micron.	
1,000,000 sq. microns	= 1 sq. millimeter.	
100 sq. millimeters	= 1 sq. centimeter.	
100 sq. centimeters	= 1 sq. decimeter.	
100 sq. decimeters	= 1 sq. meter	= 1 centare
100 sq. meters	= 1 sq. decameter	= 1 are.
100 sq. decameters	= 1 sq. hectometer	= 1 hectare.
100 sq. hectometers	= 1 sq. kilometer.	
100 sq. kilometers	= 1 sq. myriameter.	

Cubic Measure

1000 cubic millimeters	= 1 cubic centimeter.
1000 cubic centimeters	= 1 liter.
10 liters	= 1 decaliter.
100 liters	= 1 hectoliter.
1000 liters	= 1 kiloliter = 1 cu. meter = 1,000,000 c.c.

The unit of capacity is the liter and represents the volume of a kilogram of water at its maximum density, 4° C. and 760 mm. mercury pressure.

METRIC SYSTEM—*Continued*

Weight

The unit of weight is the gram and represents the weight of one cubic centimeter of water at its maximum density, 4° C. and 760 mm. mercury pressure.

10 milligrams	=1 centigram.
10 centigrams	=1 decigram.
10 decigrams	=1 gram.
10 grams	=1 decagram.
10 decagrams	=1 hectogram.
10 hectograms	=1 kilogram=1000 grams.
10 kilograms	=1 myriagram.
10 myriagrams	=1 quintal.
10 quintals	=1 millier or tonneau.

LIST OF TEXT AND REFERENCE BOOKS

Abbot, A. C. The Principles of Bacteriology. 9th Ed. 1915.

Aikman, C. M. Milk, its Nature and Composition. 1909.

American Public Health Association. Standard Methods for the Examination of Water and Sewage. 1920.

Ball, M. V. Essentials of Bacteriology. 8th Ed. 1919.

Bastian, H. C. The Beginnings of Life. 1872.

Bayliss, W. M. The Nature of Enzymic Action. 3d Ed. 1914.

Bayliss, W. M. Principles of General Physiology. 1915.

Beatty, James. The Method of Enzyme Action. 1917.

Belcher, S. D. Clean Milk. 1912.

Besson, A. Practical Bacteriology, Microbiology and Serum Therapy. Translated by H. J. Hutchens. 1913.

Bolduan, C. F., and Grund, M. Applied Bacteriology for Nurses. 1919.

Bordet, J., and Gay, F. P. Studies in Immunity. 1st Ed. 1909.

Boston, L. Napoleon. Clinical Diagnosis. 1905.

Bowhill, T. Manual of Bacteriological Technique. 2d Ed. 1902.

Brachvogel, John K. Industrial Alcohol, Its Manufacture and Uses. 1907.

Brannt, Wm. T. Vinegar, Acetates, Cider, Fruit Wines, Preservation of Fruits. 3d Ed. 1914.

Broadhurst, J. Outlines of an Elementary Course in Microbiology. 1918.

Browning, C. H. Applied Bacteriology. 1918.

Buchanan, R. E. Veterinary Bacteriology. 2d Ed. 1916.

Buchanan, R. E., and Estelle, D. Household Bacteriology. 1913.

Burgess, Paul S. Soil Bacteriology Laboratory Manual. 1914.

Calkins, Gary N. Protozoology. 1909.

Chapin, Charles V. The Sources and Modes of Infection. 2d Ed. 1912.

Chemical Rubber Co., Cleveland, Ohio. Handbook of Chemistry and Physics. 7th Ed. 1919.

Chester, F. D. A Manual of Determinative Bacteriology. 1901.
Citron, Julius. Immunity. Translated by A. L. Garbat, 1912.
Cohnheim, Otto. Enzymes. 1st Ed. 1912.
Conn, H. W. Agricultural Bacteriology. 3d Ed. 1918.
Conn, H. W. Bacteria in Milk and its Products. 1903.
Conn, H. W. Bacteria, Yeasts and Molds in the Home. 2d Ed. 1917.
Conn, H. W. Practical Dairy Bacteriology. 1910.
Conn, H. W., Esten, W. M., Stocking, W. A. Classification of Dairy Bacteria. Reprint from the Report of the Storrs Connecticut Agr'l Exp't. Sta. for 1906.
Craig, C. F. Wassermann Test. 1918.

De Bary, H. Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria. 1887.
De Bary, H. Lectures on Bacteria. 2d Ed. 1887.
Decker, John W. Cheese Making. 1905.
Dieudonne, H. Bacterial Food Poisoning. 1909.
Doane, R. W. Insects and Disease. 1910.
Don, John and Chisholm, John. Modern Methods of Water Purification. 1911.
Duckwall, E. W. Canning and Preserving of Food Products with Bacteriological Technique. 1905.
Duggar, B. M. Fungous Diseases of Plants. 1909.
Duggar, B. M. Plant Physiology. 1912.
Durck, Hermann. Allgemeine Pathologische Histologie. Teil III. 1903.

Effront, Jean. Enzymes and their Application. 1st Ed. 1904.
Ehrlich, Paul. Studies in Immunity. Translated by Chas. Bolduan. 2d Ed. 1910.
Eisenberg, A. A. Principles of Bacteriology. 1918.
Elliot, S. Maria. Household Bacteriology. 1914. From Handbook of Health and Nursing. Edited by the American School of Home Economics. 1912.
Ellis, D. Iron Bacteria. 1919.
Ellis, D. Outlines of Bacteriology. 1909.
Ernst, Wm. Milk Hygiene. Translated by John R. Mohler and Adolph Eichhorn. 1914.

Euler, Hans. General Chemistry of the Enzymes. Translated by T. H. Pope. 1st Ed. 1912.
Eyre, J. W. H. Bacteriological Technique. 2d Ed. 1913.

Farrington, E. H., and Woll, F. W. Testing Milk and its Products. 1904.
Fischer, Alfred. Structure and Functions of Bacteria. Translated by A. Coppen Jones. 1900.
Fleischmann, W. The Book of the Dairy. 1896. Translated by C. M. Aikmann and R. Patrick Wright.
Flügge, C. Die Mikroörganismen. 1896.
Fowler, G. J. Bacteriological and Enzyme Chemistry. 1911.
Fox, H. Elementary Bacteriology and Protozoology for Use of Nurses. 3d Ed. 1919.
Fred, E. B. A Laboratory Manual of Soil Bacteriology. 1916.
Frost, W. D. A Laboratory Guide in Elementary Bacteriology. 3d Ed. 1904.
Frost, W. D., and McCampbell, E. F. A Text-book of General Bacteriology. 1910.
Fuhrmann, Franz. Vorlesungen über bakterien Enzyme. 1907.
Fuhrmann, Franz. Vorlesungen über technische Mykologie. 1913.
Fuller, G. W. Sewage Disposal. 1912.

Gage, S. H. The Microscope. 11th Ed. 1911.
Gerhard, Wm. P. The Sanitation, Water Supply and Sewage Disposal of Country Houses. 1909.
Gorham, F. P. Laboratory Course in Bacteriology. 1915.
Green, J. Reynolds. Soluble Ferments and Fermentation. 2d Ed. 1901.
Gueguen, F. Les Champignons: Parasites de l'Homme and des Animaux. 1904.
Gurney, Dixon. Transmutation of Bacteria. 1919.
Guilliermond, A. Les Levures. 1912.
Guilliermond, A. The Yeasts. Translated by F. W. Tanner. 1920.
Guthrie, E. S. The Book of Butter. 1918.

Hansen, Emil Chr. Practical Studies in Fermentation. 1896.
Harden, Arthur. Alcoholic Fermentation. 2d Ed. 1914.

Hastings, E. G. and Wright, W. H. A Laboratory Manual of General Agricultural Bacteriology. 1913.
Hawk, P. B. Physiological Chemistry. 6th Ed. 1918.
Heinemann, Paul G. A Laboratory Guide in Bacteriology. 2d Ed. 1913.
Heinemann, Paul G. Milk. 1919.
Hertwig, Oscar. Allgemeine Biologie. 1912.
Herzog, M. Disease-Producing Microörganisms. 1910.
Hewlett, R. T. Manual of Bacteriology. 5th Ed. 1915.
Hiss, P. H., and Zinsser, Hans. Text-book of Bacteriology, 4th Ed. 1919.
Hooker, A. H. Chloride of Lime in Sanitation. 1913.
Hueppe, F. Methods of Bacteriological Investigation. Translated by H. M. Biggs. 1886.
Hunziker, O. F. The Butter Industry. 1920.

Jago, Wm., and Jago, Wm. C. The Technology of Bread-making. 1911.

Jensen, C. O. Essentials of Milk Hygiene. Translated by Leonard Pearson. 1907.
Jess, Paul. Kompendium der Bakteriologie und Blutserumtherapie. 1903.
Jordan, Edwin O. General Bacteriology. 6th Ed. 1918.
Jordan, Edwin O. Food Poisoning. 1917.
Jorgensen, A. Microorganism and Fermentation. Translated by A. K. Miller and E. A. Lennholm. 1893.

Kendall, A. I. Bacteriology, General, Pathological and Intestinal. 1916.
Kershaw, G. B. Modern Methods of Sewage Purification. 1911.
Kinnicutt, L. P., Winslow, C.-E. A., and Pratt, R. W. Sewage Disposal, 2d Ed. 1919.
Kisskalt, K. und Hartmann, M. Praktikum der Bakteriologie, und Protozoologie. 1907.
Kitt, Th. Bakterienkunde und pathologische Mikroskopie. 1903.
Kitt, Th. Text-book of Comparative General Pathology. Translated by Wm. W. Cadbury and Allen J. Smith. 1906.
Klein, Louis A. Principles and Practice of Milk Hygiene. 1917.

Klöcker, Alb. Fermentation Organisms. Translated by G. E. Allen and J. H. Millar. 1904.

Kölle, W., und Wassermann, A. Handbuch der pathogenen Microorganismen I, II und III und Atlas. 1903.

Kolmer, J. A. Infection, Immunity and Specific Therapy. 2d Ed. 1917.

Kraus, R., und Levaditi, C. Handbuch der Technik und Methodik der Immunitätsforschung. Bd. I, Antigene. 1908; Bd. II, Antikörper, 1909; Erster Ergänzungsband. 1911.

Kruse, W. Allgemeine Mikrobiologie. 1910.

Lafar, Franz. Die Essigsäure-Gärung. 1913.

Lafar, Franz. Technische Mykologie. German Ed. Bd. I; 1904–1907; Bd. II, 1905–1908; Bd. III, 1904–1906; Bd. IV, 1905–1907; Bd. V, 1905–1914. English Ed. Vol. I, 1898, Vol. II, Part I, 1903; Vol. II, Part II, 1910. Translated by Charles T. C. Salter.

Law, James. Veterinary Medicine. 3d Ed. Vol. I. 1910; Vol. II, 1911.

Lee, H. Bolles. The Microtomist's Vade Mecum. 6th Ed. 1905.

Lefas, E. La Technique histo-bacteriologique moderne. 1906.

Lehmann, K. B., u. Neumann, R. O. Bakteriologie und bakteriologische Diagnostik. Teil I, Atlas, 1910; Teil II, Text, 1912.

Lipman, J. G. Bacteria in Relation to Country Life. 1908.

Lipman, J. G., and Brown, P. E. Laboratory Guide in Soil Bacteriology. 1911.

Loeb, Jacques. The Mechanistic Conception of Life. 1912.

Loeb, Jacques. Studies in General Physiology, Part I. 1905.

Löhnis, F. Handbuch der landwirtschaftliche Bakteriologie. 1910.

Löhnis, F. Laboratory Methods in Agricultural Bacteriology. Translated by Wm. Stevenson and J. Hunter Smith. 1913.

Löhnis, F. Vorlesungen über landwirtschaftliche Bakteriologie. 1913.

MacFadyean, Allan. The Cell as a Unit of Life. 1908.

MacNeal, W. J. Pathogenic Microorganisms. 1920.

Marshall, C. E. Microbiology. 3d Ed. 1921.

MASON, W. P. Examination of Water, Chemically and Bacteriologically. 5th Ed. 1917.
MASSEE, G. Diseases of Cultivated Plants and Trees. 1914.
MAST, S. O. Light and the Behavior of Organisms. 1911.
MATTHEWS, C. G. Manual of Alcoholic Fermentation. 1901.
MCCAMPBELL, E. F. Laboratory Methods for the Experimental Study of Immunity. 1909.
MCCONNEL, G. General Pathology and Bacteriology for Dental Students. 2d Ed. 1918.
MCFARLAND, JOSEPH. Pathogenic Bacteria and Protozoa. 9th Ed. 1919.
MCFARLAND, JOSEPH. Biology, General and Medical. 2d Ed. 1914.
MCISAAC, ISABEL. Bacteriology for Nurses. 1913.
MCKAY, G. L., and LARSEN, C. Principles and Practice of Buttermaking. 1913.
MERCK'S INDEX. 1907.
METCALF, L., and EDDY, H. P. American Sewerage Practice. 2d Ed. 1916.
METCHNIKOFF, ELIE. Comparative Pathology of Inflammation. Translated by F. A. Starling and E. H. Starling. 1893.
METCHNIKOFF, ELIE. Immunity in Infective Diseases. 1905.
MIGULA, W. System der Bakterien Bd. I, 1897; Bd. II, 1900.
MOLISCH, HANS. Die Eisenbakterien. 1910.
MOLISCH, HANS. Leuchtende Pflanzen. 1904.
MOLISCH, HANS. Die Purpurbakterien. 1907.
MOORE, V. A. The Pathology and Differential Diagnosis of Infectious Diseases of Animals. 4th Ed. 1916.
MOORE, V. A. Principles of Microbiology. 1916.
MOORE, V. A. Bovine Tuberculosis and its Control. 1913.
MOORE, V. A., and FITCH, C. P. Bacteriology and Diagnosis. 1914.
MORSE, M. E. First Lessons in Bacteriology for Nurses. 1919.
MORREY, C. B. Fundamentals of Bacteriology. 1917.
MUIR, ROBERT, and RITCHIE, JAMES. Manual of Bacteriology. 7th Ed. 1919.
MÜLLER, PAUL TH. Vorlesungen über Infektion und Immunität. 1904.

Nocard, Ed., and Leclainche, E. Les Maladies microbiennes des Animaux T. I, 1903; T. II, 1903.
Novy, F. G. Laboratory Work in Bacteriology. 1899.
Nuttall, G. H. F. Blood Immunity and Blood Relationship; Precipitin Tests. 1904.

Ogden, Henry N., and Cleveland, H. Burdett. Practical Methods of Sewage Disposal. 1912.
Oppenheimer, C. Die Fermente und ihre Wirkungen. Bd. I, Vierte Auflage. 1913; Bd. II, 1913.

Park, Wm. H. Pathogenic Microorganisms. 7th Ed. 1920.
Park, Wm. H. Public Health and Hygiene. 1920.
Parker, H. M. Milk Supplies. 1917.
Pasteur, L. Studies on Fermentations. Translated by F. Faulkner and D. C. Robb. 1879.
Percival, J. Agricultural Bacteriology, Theoretical and Practical. 1920.
Pfeiffer, L. Die Protozoen als Krankheitserreger. 1890.
Pitfield, R. L. Compend on Bacteriology, including Pathogenic Protozoa. 3d Ed. 1917.
Porcher, C. H. Le Lait desseche. 1912.
Prescott, S. C. and Winslow, C.-E. A. Elements of Water Bacteriology. 3d Ed. 1913.
Prudden, T. M. The Story of the Bacteria. 1917.

Race, Joseph. Chlorination of Water. 1918.
Race, Joseph. Examination of Milk for Public Health Purposes. 1918.
Richmond, H. D. Dairy Chemistry. 1899.
Ricketts, H. T., and Dick, G. F. Infection, Immunity and Serum Therapy. 1913.
Rideal, Samuel. Sewage and the Bacterial Purification of Sewage. 1901.
Roberts, J. G. Manual of Bacteriology and Pathology for Nurses. 3rd Ed. 1920.
Roddy, J. Medical Bacteriology. 1917.
Roechling, H. H. Sewer Gas and its Influence upon Health. 1898.

Rogers, A., and Aubert, A. B. Industrial Chemistry. 1912.
Rosenau, M. J. Disinfection and Disinfectants. 1902.
Rosenau, M. J. The Milk Question. 1912.
Rosenau, M. J. Preventive Medicine and Hygiene. 3d Ed. 1917.
Russell, H. L., and Hastings, E. G. Experimental Dairy Bacteriology. 10th Ed. 1914.
Russell, H. L., and Hastings, E. G. Outlines of Dairy Bacteriology. 11th Ed. 1920.
Russell, H. L., and Hastings, E. G. Agricultural Bacteriology. 1921.

Sadtler, S. P. Industrial Organic Chemistry. 1912.
Santee, E. M. Farm Sewage. 1912.
Savage, Wm. G. The Bacteriological Examination of Water Supplies. 1906.
Savage, Wm. G. The Bacteriological Examination of Food and Water. 2d Ed. 1916.
Savage, Wm. G. Milk and the Public Health. 1920.
Schneider, Albert. Bacteriological Methods in Food and Drug Laboratories. 1915.
Schneider, Albert. Pharmaceutical Bacteriology. 2d Ed. 1920.
Scheidner, Albert. Microbiology and Microanalysis of Foods. 1920.
Sherman, G. H. Vaccine Therapy in General Practice. 1916.
Shinkle, C. A. American Commercial Methods of Manufacturing Preserves, Pickles, Canned Foods, etc. Revised Ed. 1912.
Simon, C. E. Human Infection Carriers. 1919.
Simon, C. E. Infection and Immunity. 3d Ed. 1915.
Smeeton, M. A. Bacteriology for Nurses. 1920.
Smith, Erwin F. Bacteria in Relation to Plant Diseases. Vols. I, 1905; II, 1911; III, 1914.
Smith, Erwin F. Bacterial Diseases of Plants. 1920.
Snyder, Harry. Dairy Chemistry. 1911.
Sternberg, G. M. Text-book of Bacteriology. 1896.
Stevens, F. L., and Hall, J. G. Diseases of Economic Plants. 1913.
Stevens, F. L. The Fungi which Cause Plant Disease. 1913.

Stitt, E. R. Practical Bacteriology, Blood Work and Animal Parasitology. 6th Ed. 1920.

Sykes, W. J. The Principles and Practice of Brewing. 1897.

Tanner, F. W. Bacteriology and Mycology of Foods. 1919.

Thom, Charles, and Fisk, W. W. The Book of Cheese. 1918.

Thomas, B. A., and Ivy, R. H. Applied Immunology, the Practical Applications of Sera and Bacterins, etc. 2d Ed. 1916.

Thorp, F. H. Outlines of Industrial Chemistry. 1905.

Thresh, John C. Examination of Water and Water Supplies. 2d Ed. 1913.

Tyndall, John. The Floating Matter of the Air in Relation to Putrefaction and Infection. 1881.

United States Army Laboratory Methods. War Manual No. 6. 1918.

Van Slyke, L. L., and Publow, C. A. The Science and Practice of Cheese-making. 1912.

Vaughn, V. C., and Novy, F. G. Cellular Toxins. 4th Ed. 1902.

Vaughn, V. C., Vaughn, V. C., Jr., and Vaughn, J. W. Protein Split Products in Relation to Immunity and Disease. 1913.

Vedder, E. B. Syphilis and Public Health. 1918.

Vernon, H. M. Intracellular Enzymes. 1908.

Walker, E. W. Ainley. Inflammation, Infection and Fever. 1904.

Warbasse, J. P. The Conquest of Disease through Animal Experimentation. 1910.

Ward, A. R. Pure Milk and the Public Health. 1909.

Ward, H. Marshall. Diseases in Plants. 1901.

Wassermann, A. Immune Sera. Translated by Chas. Baldwin. 1904.

Webster, R. W. Diagnostic Methods. 5th Ed. 1916.

Whipple, G. C. Typhoid Fever. 1st Ed. 1908.

Whipple, G. C. The Microscopy of Drinking Water. 3rd Ed. 1914.

Whiting, A. L. Soil Biology. 1917.

WILLIAMS, H. U. Manual of Bacteriology. Revised by B. Meade Bolton. 5th Ed. 1908.

WILLOUGHBY, EDWARD F. Milk: Its Production and Uses. 1903.

WILSON, EDMUND B. The Cell in Development and Inheritance. 1911.

WING, HENRY H. Milk and its Products. 1904.

WINSLOW, C.-E. A., and WINSLOW, ANNE R. The Systematic Relationship of the Coccaceæ. 1908.

WOODMAN, A. G., and NORTON, J. F. Air, Water and Food. 4th Ed. 1914.

ZAVALLA, JUSTO P. The Canning of Fruits and Vegetables. 1916.

ZIEGLER, ERNST. Pathologie und Anatomie. Bd. I, 1901; Bd. II, 1902.

ZINSSER, HANS. Infection and Resistance. 2d Ed. 1918.

ZINSSER, HANS, HOPKINS, J. G., and OTTENBURG, R. A Laboratory Course in Serum Study. 1916.

ZOPF, WILHELM. Die Pilze. 1890.

PERIODICALS

Abstracts of Bacteriology.

Annales de l'Institut Pasteur.

Arbeiten aus dem kaiserlichen Gesundheitsamte.

Archiv für experimentale Pathologie und Pharmakologie.

Archiv für Hygiene.

Archiv für Schiffs- und Tropenhygiene.

Berliner klinische Wochenschrift.

Biochemische Zeitschrift.

British Medical Journal.

Bulletin de l'Institut Pasteur.

Centralblatt für Bakteriologie, I Abteilung, Originale. Medizinisch-hygienische Bakteriologie und tierische Parasitenkunde.

Centralblatt für Bakteriologie, I Abteilung, Referate.

Centralblatt für Bakteriologie, II Abteilung. Allgemeine landwirtschaftlich-technologische Bakteriologie, Gärungs-physiologie und Pflanzenpathologie.

Chemical Abstracts.

Comptes Rendus Académie des Sciences.

Comptes Rendus Sociétié de Biologie.
Comptes Rendus des Travaux du Laboratoire de Carlsberg.

Deutsche medizinische Wochenschrift.

Experiment Station Record.

Hygienische Rundschau.

Jahresbericht über die Forsch. d. path. Mikroorganismen, Baumgarten's.
Jahresbericht über die Fortschritte der Lehre von den Gärungs Organismen, Koch's.
Journal of Agricultural Research.
Journal of the American Medical Association.
Journal of the American Public Health Association.
Journal of the American Veterinary Medical Association.
Journal of Bacteriology.
Journal of Clinical and Laboratory Medicine.
Journal of Dairy Science.
Journal of Experimental Medicine.
Journal of Hygiene.
Journal of Industrial Hygiene.
Journal of Industrial and Engineering Chemistry.
Journal of Infectious Disease.
Journal of Immunology.
Journal of Medical Research.
Journal of Pathology and Bacteriology.
Journal of the Royal Army Medical Corps.
Journal of Tropical Medicine.

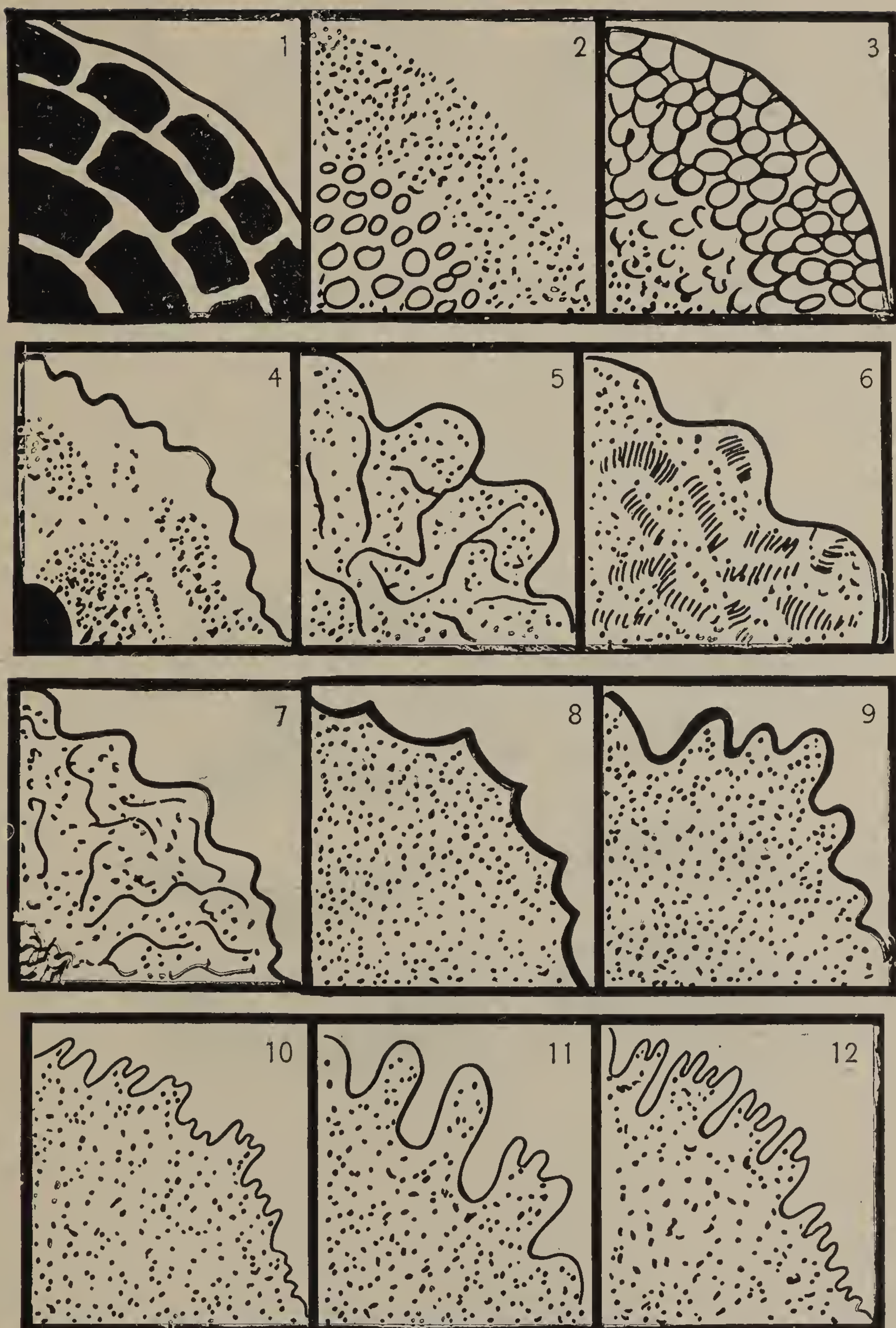
Lancet.

Proceedings of the Royal Society of London.

Revue générale du Lait.

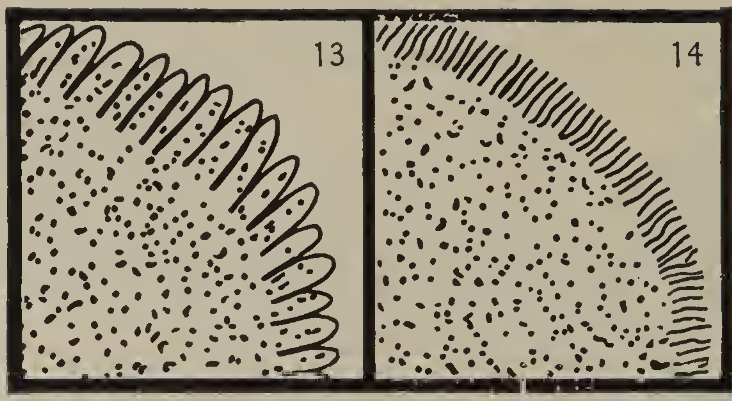
Zeitschrift für Hygiene und Infektionskrankheiten.
Zeitschrift für Immunitätsforschung.

A

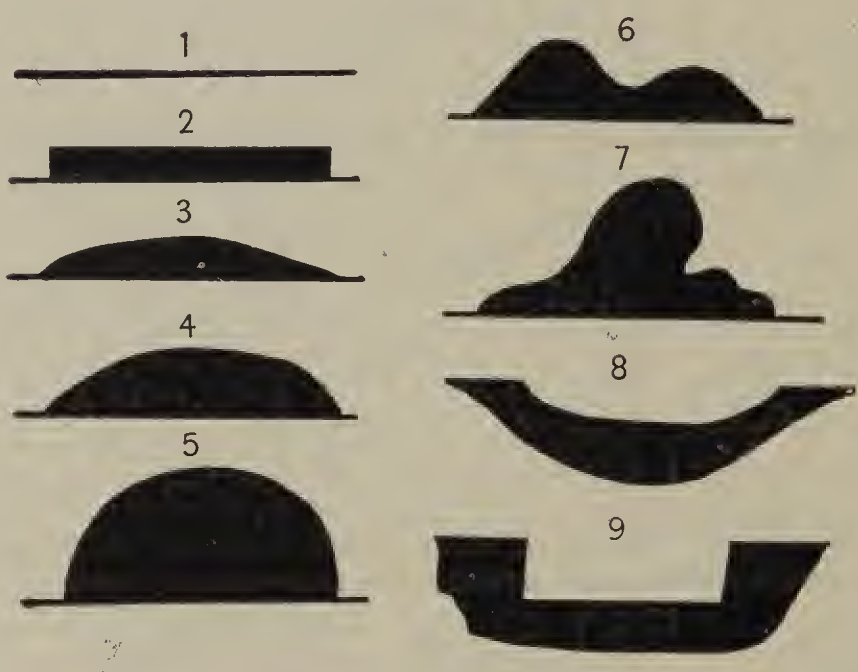


Structure of Colonies.	Edges of Colonies.
1. Areolate.	8. Repand.
2. Grumose.	9. Lobate.
3. Moruloid.	10. Erose.
4. Clouded.	11. Auriculate.
5. Gyrose.	12. Lacerate.
6. Marmorate.	13. Fimbriate.*
7. Reticulate.	14. Ciliate.*

* For illustration see next page.



B



Elevation of Colonies.

1. Flat.
2. Raised.
3. Convex.
4. Pulvinate.
5. Hemispherical.
6. Umbilicate.
7. Umbonate.
8. Concave.
9. Rectangular depression.

FORM OF COLONIES.

1. Round.
2. Irregular.
3. Elliptical.
4. Spindle.
5. Mycelioid.
6. Filamentous.
7. Rhizoid.
8. Cochleate.
9. Conglomerate.
10. Rosulate.
11. Toruloid.
12. Curled.

*D**.

STAB CULTURES.

1. Filiform.
2. Beaded.
3. Papillate.
4. Villous.
5. Arborescent.

E.*

TYPES OF LIQUEFACTION.

1. Crateriform.
2. Napiform.
3. Infundibuliform.
4. Saccate.
5. Stratiform.

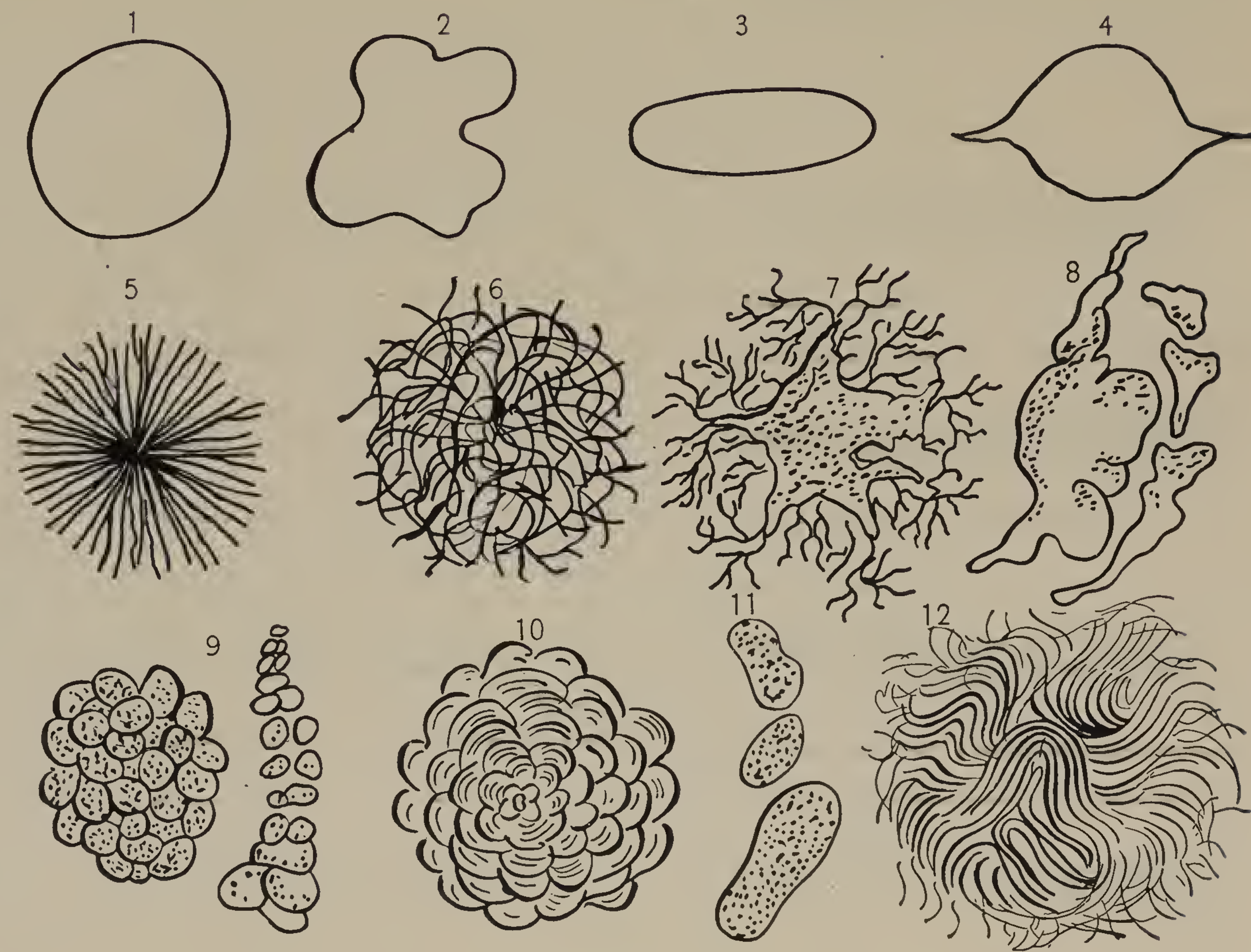
F.*

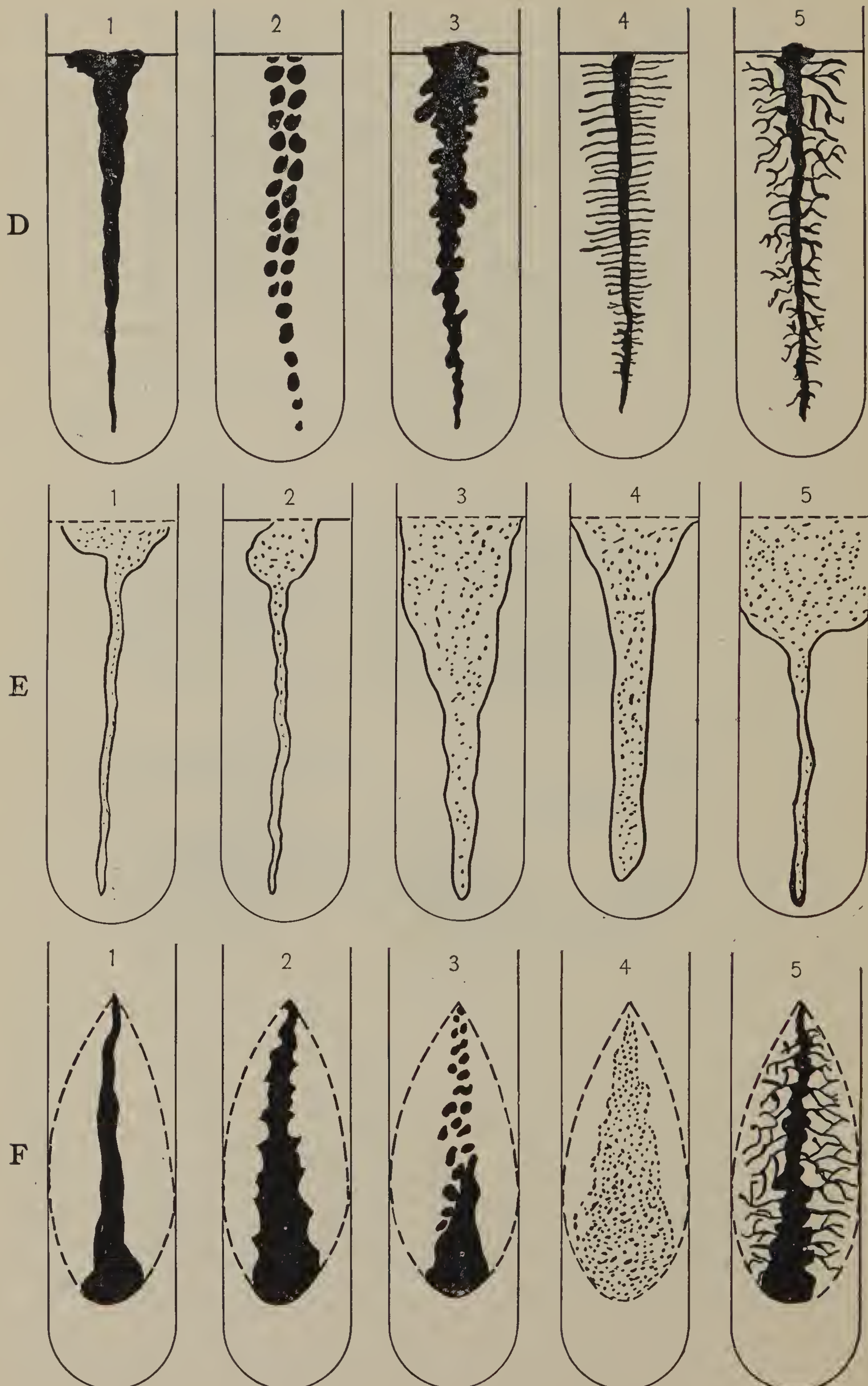
STREAK CULTURES.

1. Filiform.
2. Echinulate.
3. Beaded.
4. Spreading.
5. Arborescent.

* For illustrations see next page.

C




1
2
3
4
5
D
1
2
3
4
5
E
1
2
3
4
5
F

INDEX

G

M

THE WILEY TECHNICAL SERIES

EDITED BY

JOSEPH M. JAMESON

A series of carefully adapted texts for use in technical vocational and industrial schools. The subjects treated will include Applied Science; Household and Agricultural Chemistry; Electricity; Electrical Power and Machinery; Applied Mechanics; Drafting and Design; Steam; Gas Engines; Shop Practice; Applied Mathematics; Agriculture; Household Science, etc.

The following texts are announced; others are being added rapidly:

ELECTRICITY

THE ELEMENTS OF ELECTRICITY; For Technical Students. By W. H. Timbie, Associate Professor of Electrical Engineering, Massachusetts Institute of Technology. xiii+554 pages, 5¼ by 8. 415 figures. Cloth, $2.75 *net.*

ANSWERS TO PROBLEMS IN ELEMENTS OF ELECTRICITY. 5 by 7¼. Paper cover, 25 cents *net.*

THE ESSENTIALS OF ELECTRICITY; A Text-book for Wiremen and the Electrical Trades. By W. H. Timbie, Massachusetts Institute of Technology. Flexible covers, pocket size. xiii+271 pages, 5 by 7¼. 224 figures. Cloth, $1.75 *net.*

ANSWERS TO PROBLEMS IN ESSENTIALS OF ELECTRICITY. 5 by 7¼. Paper cover, 25 cents *net.*

CONTINUOUS AND ALTERNATING CURRENT MACHINERY. By J. H. Morecroft, Associate Professor of Electrical Engineering, Columbia University. ix+466 pages, 5¼ by 8. 288 figures. Cloth, $2.75 *net.*

8/30/21

CONTINUOUS AND ALTERNATING CURRENT MACHINERY PROBLEMS. By W. T. RYAN, E.E., Assistant Professor of Electrical Engineering, The University of Minnesota. vii+37 pages, 5¼ by 8. Cloth, 50 cents *net.*

ALTERNATING CURRENT ELECTRICITY AND ITS APPLICATION TO INDUSTRY. By W. H. TIMBIE, Associate Professor of Electrical Engineering, Massachusetts Institute of Technology, and H. H. HIGBIE, Professor of Electrical Engineering, University of Michigan. **First Course.** x+534 pages, 5¼ by 8. 389 figures. Cloth, $3.50 *net.*
Second Course. ix+729 pages. 5¼ by 8. 357 figures. Cloth, $4.00 *net.*

ANSWERS TO PROBLEMS IN ALTERNATING CURRENT ELECTRICITY. First and Second Courses. 5 by 7¼. Paper cover, 50 cents *net.*

ESSENTIALS OF ALTERNATING CURRENTS. By W. H. TIMBIE and H. H. HIGBIE. viii+374 pages. 5 by 7. 223 figures. Cloth, $2.25 *net.*

ANSWERS TO ESSENTIALS OF ALTERNATING CURRENTS. 5 by 7¼. Paper cover, 25 cents *net.*

HEAT AND HEAT ENGINEERING

HEAT; A Text-book for Technical and Industrial Students. By J. A. RANDALL, formerly Instructor in Mechanics and Heat, Pratt Institute. xiv+331 pages, 5¼ by 8. 80 figures. Cloth, $2.00 *net.*

GAS POWER. By C. F. HIRSHFELD, formerly Professor of Power Engineering, Sibley College, Cornell University, and T. C. ULBRICHT, formerly Instructor, Department of Power Engineering, Cornell University. x+209 pages, 5¼ by 8. 60 figures. Cloth, $1.75 *net.*

STEAM POWER. By C. F. HIRSHFELD, formerly Professor of Power Engineering, Sibley College, Cornell University, and T. C. ULBRICHT, formerly Instructor, Department of Power Engineering. Cornell University. viii+420 pages, 5¼ by 8. 228 Figures. Cloth, $3 00 *net.*

MECHANICS AND MATHEMATICS

ELEMENTARY PRACTICAL MECHANICS. By J. M. JAMESON, Girard College, formerly of Pratt Institute. xii+321 pages, 5 by 7¼. 212 figures. Cloth, $1.75 *net.*

MATHEMATICS FOR MACHINISTS. By R. W. BURNHAM, Chief Coordinator, Bureau of Vocational Activities, Department of Education, New York City. vii+229 pages, 5 by 7. 175 figures. Cloth, $1.75 *net.*

PRACTICAL SHOP MECHANICS AND MATHEMATICS. By JAMES F. JOHNSON, formerly Superintendent of the State Trade School, Bridgeport, Conn. viii+130 pages, 5 by 7. 81 figures. Cloth, $1.40 *net.*

PRINCIPLES OF MECHANISM. By WALTER H. JAMES, Assistant Professor of Mechanical Engineering Drawing, Massachusetts Institute of Technology, and MALCOLM C. MACKENZIE, Instructor in Mechanical Engineering, Massachusetts Institute of Technology. v+241 pages, 5¼ by 7½. 244 figures. Cloth, $2.00 *net.*

ARITHMETIC FOR CARPENTERS AND BUILDERS. By R. BURDETTE DALE, formerly Director in charge of Vocational Courses in Engineering and Correspondence Instruction, Iowa State College. ix+231 pages, 5 by 7. 109 figures. Cloth, $1.75 *net.*

MATHEMATICS FOR SHOP AND DRAWING STUDENTS. By H. M. KEAL, Head of Department of Mathematics, and C. J. LEONARD, Instructor in Mathematics, Cass Technical High School, Detroit, Michigan. vii+213 pages, 4⅞ by 7. 188 figures. Cloth, $1.60 *net.*

MATHEMATICS FOR ELECTRICAL STUDENTS. By H. M. KEAL and C. J. LEONARD. vii+230 pages, 4⅞ by 7. 165 figures. Cloth, $1.60 *net.*

PREPARATORY MATHEMATICS FOR USE IN TECHNICAL SCHOOLS. By HAROLD B. RAY and ARNOLD V. DOUB, Instructors in Mathematics, Cass Technical High School, Detroit, Michigan. vii+68 pages, 4⅞ by 7. 70 figures. Cloth, $1.00 *net.*

SHOP TEXTS

MACHINE SHOP PRACTICE. By W. J. KAUP, Consulting Engineer. xii+198 pages, 5¼ by 8. 163 figures. Cloth, $1.75 *net.*

PATTERN MAKING. By FREDERICK W. TURNER and DANIEL G. TOWN, Mechanic Arts High School, Boston. v+114 pages, 5 by 7. 88 figures. Cloth, $1.00 *net.*

PLAIN AND ORNAMENTAL FORGING. By ERNST SCHWARZKOPF. Instructor at Stuyvesant High School, New York City. x+267 pages, 5¼ by 8. Over 400 figures. Cloth, $2.00 *net.*

CONCRETE

CONCRETE WORK. By WILLIAM K. HATT, C.E., Ph.D., Professor of Civil Engineering, Purdue University, and WALTER C. VOSS, B.S., Head, Department of Architectural Construction, Wentworth Institute, Boston, Mass. Vol. I, xix+451 pages, 5¼ by 7⅝, 224 figures and 20 full-page plates. Cloth, $4.00 *net.* Vol. II, xiv+206 pages, 5¼ by 7⅝, 37 figures, 97 job sheets. Cloth, $2.00 *net.*

DRAFTING AND DESIGN

DECORATIVE DESIGN. A Text-Book of Practical Methods. By JOSEPH CUMMINGS CHASE, Instructor in Decorative Design at the College of the City of New York and at Cooper Union Woman's Art School. vi+73 pages. 8 by 10¾. 340 figures. Cloth, $2.25 *net.*

AGRICULTURAL DRAFTING. By CHARLES B. HOWE, M.E. viii+63 pages, 8 by 10¾. 45 figures, 26 plates. Cloth $2 00 *net.*

ARCHITECTURAL DRAFTING. By A. B. GREENBERG, Stuyvesant Technical High School, New York, and CHARLES B. HOWE, Bushwick Evening High School, Brooklyn. viii+110 pages, 8 by 10¾. 53 figures, 12 plates. Cloth, $2.00 *net.*

MECHANICAL DRAFTING. By CHARLES B. HOWE, M.E., Bushwick Evening High School, Brooklyn. x+147 pages, 8 by 10¾. 165 figures, 38 plates. Cloth, $2.50 *net.*

DRAWING FOR BUILDERS. By R. BURDETTE DALE, formerly Director of Vocational Course, Iowa State College. v+166 pages, 8 by 10¾. 69 figures, 50 plates. Cloth, $2.50 *net.*

ELEMENTS OF MACHINE DESIGN. By HENRY L. NACHMAN, Associate Professor of Kinematics and Machine Design, Armour Institute of Technology. v+245 pages, 6 by 9. 269 figures. Cloth, $2.25 *net.*

AGRICULTURE AND HORTICULTURE

FIELD AND LABORATORY STUDIES OF SOILS. By Professor A. G. McCall, Maryland Agricultural College. viii+77 pages, 5 by 7. 32 figures. Cloth, 75 cents *net.*

FIELD AND LABORATORY STUDIES OF CROPS. By Professor A. G. McCall, Maryland Agricultural College. viii+133 pages, 5 by 7. 54 figures. Cloth, $1.00 *net.*

MARKET GARDENING. By F. L. Yeaw, Oasis Farm & Orchard Company, Roswell, New Mexico. Formerly Professor of Market Gardening, Massachusetts Agricultural College. vi+120 pages, 5 by 7. 36 figures. Cloth, $1.00 *net.*

THE CHEMISTRY OF FARM PRACTICE. By T. E. Keitt, Chemist of South Carolina Experiment Station, and Professor of Soils, Clemson Agricultural College. xii+253 pages, 5¼ by 8. 81 figures. Cloth, $2.00 *net.*

STUDIES OF TREES. By J. J. Levison, Formerly Forester, Park Department, Brooklyn, N. Y. x+253 pages, 5¼ by 8. 156 half-tone illustrations. Cloth, $2.00 *net.*

AGRICULTURAL DRAFTING. By Charles B. Howe, M.E. 63 pages, 8 by 10¾. 45 figures, 26 plates. Cloth, $2.00 *net.*

SCHOOL ENTOMOLOGY. For Secondary Schools and Agricultural Short Courses. By E. Dwight Sanderson, Formerly Dean, College of Agriculture, West Virginia University, and L. M. Peairs, Professor of Entomology, West Virginia University. vii+356 pages, 6 by 9. 233 figures. Cloth, $2.50 *net.*

BIOLOGY

LABORATORY MANUAL IN GENERAL MICROBIOLOGY. Prepared by the Laboratory of Bacteriology, Hygiene and Pathology, Michigan Agricultural College, Ward Giltner, Head of Department. Second Edition, Revised and Enlarged. xvi+469 pages, 5¼ by 8. 77 figures. Several tables and charts. Cloth, $3.50 *net.*

COSTUME DESIGNING

COSTUME DESIGN AND ILLUSTRATION. By Ethel H. Traphagen, Instructor and Lecturer at Cooper Union, The New York Evening School of Industrial Art. ix+199 pages, 8 by 10¾. Upwards of 200 illustrations, including several in colors and a color spectrum chart. Cloth, $3.50 *net.*

STUDENTS' MANUAL OF FASHION DRAWING. Thirty Lessons with Conventional Charts. By EDITH YOUNG, Director of the Edith Young Art School, Newark, N. J., formerly Art Director of the Albert Studio of Fashion Drawing, Albert Business College, Newark, N. J., formerly Instructor in Fashion Drawing at the Young Women's Christian Association, Newark, N. J. vii+106 pages. 8 by 10¾. 30 full-page plates of original drawings. Cloth, $2.50 *net*.

PRINTING

PRINTING. A Textbook for Printers' Apprentices, Continuation Classes and for General Use in Schools. By FRANK S. HENRY, Instructor in Printing, Philadelphia Trades School. ix+318 pages. 5¼ by 7⅝. 153 figures. Cloth, $1.50 *net*.

DOMESTIC SCIENCE

FOOD: ITS COMPOSITION AND PREPARATION. A Textbook for Classes in Household Science. By MARY T. DOWD and JEAN D. JAMESON, Teachers in Household Science, Washington Irving High School, New York City. vii+173 pages, 5¼ by 8. 42 figures. Cloth, $1.50 *net*.

THE LOOSE LEAF LABORATORY MANUAL

A series of carefully selected exercises to accompany the texts of the Series, covering every subject in which laboratory or field work may be given. Each exercise is complete in itself, and is printed separately. 8 by 10½.

Important Notice

WILEY LOOSELEAF MANUALS

The sale of separate sheets of the Laboratory Manuals of the Wiley Technical Series has been discontinued. These Manuals will, hereafter, be sold only as a complete book with removable leaves. Descriptive literature will be sent on request.

CHEMISTRY

Exercises in General Chemistry. By CHARLES M. ALLEN, Head of Department of Chemistry, Pratt Institute. An introductory course in Applied Chemistry, covering a year's laboratory work on the acid-forming and metallic elements and compounds. 62 pages, 8 by 10½. 61 exercises.

Complete in paper cover. Removable leaves. $1.25 *net*.

Quantitative Chemical Analysis. By Charles M. Allen, Head of Department of Chemistry, Pratt Institute. 12 pamphlets. 8 by 10½. Complete in paper cover. Removable leaves. $1.00 *net.*

Qualitative Chemical Analysis. By C. E. Bivins, Instructor in Qualitative Analysis, Pratt Institute. 11 pamphlets, supplemented by *Work Sheets* by which the student is taught equations and chemical processes. Complete with work sheets in paper cover. Removable leaves. $1.50 *net.*

Technical Chemical Analysis. By R. H. H. Aungst, Instructor in Technical Chemistry, Pratt Institute. 19 pamphlets. 8 by 10½. Complete. Removable leaves. $1.00 *net.*

MECHANICS AND HEAT

Exercises in Mechanics. By J. M. Jameson, Girard College; Formerly of Pratt Institute. 52 exercises. Complete in paper cover. Removable leaves. 85 cents *net.*

Exercises for the Applied Mechanics Laboratory. Steam; Strength of Materials; Gas Engines; and Hydraulics. By J. P. Kottcamp, M.E., Instructor in Steam and Strength of Materials, Pratt Institute. 8 by 10½. 58 exercises, with numerous cuts and tables. Complete in paper cover. Removable leaves. $1.00 *net.*

ELECTRICITY

Exercises in Heat and Light. By J. A. Randall, Instructor in Mechanics and Heat, Pratt Institute. 17 exercises, with numerous cuts and diagrams. 8 by 10½. Complete in paper cover. Removable leaves. 34 cents *net.*

Electrical Measurements, A. C. and D. C. By W. H. Timbie, Associate Professor of Electrical Engineering, Massachusetts Institute of Technology. 52 Exercises. Complete in paper cover, 85 cents *net.*

Elementary Electrical Testing. By Professor V. Karapetoff. Cornell University, Ithaca, N. Y. 25 exercises. Complete in paper cover. Removable leaves. 50 cents *net.*

Electrical Measurements and Testing. (*Direct* and *Alternating Current.*) By CHESTER L. DAWES, Instructor in Electrical Engineering, Harvard University. In charge of Industrial Electricity, Franklin Union, Boston. 39 Exercises. Complete in paper cover. Removable leaves. 75 cents *net.*

AGRICULTURE AND HORTICULTURE

Studies of Trees: Their Diseases and Care. By J. J. LEVISON, M.F., Lecturer on Ornamental and Shade Trees, Yale University Forest School, formerly Forester to the Department of Parks, Brooklyn, N.Y. 20 pamphlets, 8 by 10½. $1.00 *net.* A cloth binder for above sold separately. 50 cents *net.*

Exercises in Farm Dairying. By Professor C. LARSEN, Department of Dairy Husbandry, South Dakota State College. Loose leaf. 8 by 10½. 69 Exercises. Complete. Removable leaves. $1.00 *net.*

DRAWING

AGRICULTURAL DRAFTING PROBLEMS. By CHARLES B. HOWE, M.E. A Manual for Students of Agriculture to Supplement the Text in Agricultural Drafting. 26 plates. 8 by 10½. In paper cover. Removable leaves. 50 cents *net.*

THE ORDERS OF ARCHITECTURE. By A. BENTON GREENBERG, A Manual for Students of Architecture to Supplement the Text in Architectural Drafting. 20 plates. 8 by 10½. In paper cover. Removable leaves. 50 cents *net.*

GENERAL DRAFTING PROBLEMS. By CHARLES B. HOWE, M.E. A series of 23 sheets. 8 by 10½. In paper cover. Removable leaves. 50 cents *net.*

MECHANICAL DRAFTING MANUAL. By CHARLES B. HOWE, M.E. A Series of Lessons and Exercises Based upon the Fundamental Principles of Drafting. 8½ by 6½. In heavy paper envelopes. Part I: 15 Lessons. General Principles of Drafting and Working Drawings. 50 cents *net.* Part II: 16 plates. Geometry of Drawing. 50 cents *net.* Complete, $1.00 *net.*